THE ROLE OF NEUROTRANSMITTERS IN THE REGULATION OF CELL PROLIFERATION IN THE EMBRYONIC CHICK RETINA

RACHAEL A PEARSON

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For my parents.
Abstract

This thesis investigates the roles of neurotransmitters and their receptors, changes in intracellular calcium ([Ca^{2+}]_i), and the retinal pigment epithelium (RPE) in the regulation of cell proliferation and migration in the developing chick retina.

Confocal imaging of retinæ is used to show that before synapses are formed, cells in the ventricular zone (VZ) display intermittent spontaneous [Ca^{2+}]_i transients that depend upon the endogenous release of neurotransmitters. Purinergic and muscarinic receptor-evoked transients occur in a mixed population of interphase and mitotic cells. Those produced by GABAergic and glutamatergic receptors are mostly restricted to the interphase population. Muscarinic receptor activation is shown to slow down, and purinergic activation to speed up, mitosis. These actions may result from the [Ca^{2+}]_i transients these agonists evoke. GABA and glutamate receptor activation are without effect on mitosis.

The nuclei of retinal progenitor cells (PCs) migrate back-and-forth across the retina in a process called interkinetic nuclear migration (INM). To study the possible influence of neurotransmitter receptor activation on INM, a 'gene gun' technique was used to label cells in the VZ; the speed of movement of some cells is influenced by neurotransmitters. [Ca^{2+}]_i transients occur in cells during INM, which may be important in its regulation.

Gap junctional communication between the RPE and the neural retina was investigated. Ca^{2+}-imaging experiments show that gap junctions support the spread of spontaneous Ca^{2+} signals between neighbouring cells. Whole-cell patch clamp recording was used to fill VZ cells with a combination of gap junction-permeable and impermeable dyes. These injections show that gap junctions couple PCs into clusters that largely exclude differentiated neurons. Coupling was also observed between cells in the RPE and the neural retina. These pathways may be important in the regulation of proliferation. The RPE is shown to express both purinergic and muscarinic receptors and to have a profound influence on the rate of cell proliferation in the neural retina. The RPE may speed mitosis in the retina through the release of ATP and other factors.
CONTENTS

Dedication ................................................................................................................ 2
Abstract ................................................................................................................... 3
Contents .................................................................................................................. 4
List of Figures ........................................................................................................ 11
Acknowledgements ................................................................................................ 15

Chapter 1
Introduction

1.1 Organisation of the retina ................................................................. 16
1.2 Information processing in the retina .................................................. 17
1.3 The chick retina as a model for developmental study ...................... 19
1.4 Development of the retina ................................................................. 19
    1.4.1 Early development of the eye
    1.4.2 The cell cycle
    1.4.3 Cell birth during retinal development
    1.4.4 Cell death during retinal development
    1.4.5 Symmetrical and asymmetrical division of progenitor cells
    1.4.6 Are radial glial cells progenitor cells?
1.5 Extrinsic and intrinsic mechanisms regulate cell proliferation
    and differentiation in the retina ................................................................. 29
1.6 Classification and localization of neurotransmitter receptors .......... 30
    1.6.1 Cholinergic neurotransmission
    1.6.2 Purinergic neurotransmission
    1.6.3 GABAergic neurotransmission
    1.6.4 Glutamatergic neurotransmission
1.7 Roles of neurotransmitters in development ......................................... 42
1.8 Spontaneous $[\text{Ca}^{2+}]_i$ transients and the regulation of neuronal
    development ................................................................................................. 44
    1.8.1 $[\text{Ca}^{2+}]_i$ stores and release mechanisms
    1.8.2 $[\text{Ca}^{2+}]_i$ and regulation of the cell cycle
    1.8.3 Spontaneous $[\text{Ca}^{2+}]_i$ activity in the developing nervous system.
1.9 Neuronal migration in development ....................................................... 50
Chapter 2

Methods

2.1 Preparation of retinal tissue ................................................................. 65
2.2 Superfusion ...................................................................................... 65
2.3 Preparation of drugs ......................................................................... 66
2.4 Ca\(^{2+}\) Imaging ............................................................................... 66
   2.4.1 Loading of retinas with Ca\(^{2+}\) sensitive dyes
   2.4.2 Imaging data acquisition, storage and analysis
   2.4.3 Calibration of [Ca\(^{2+}\)]\(_{i}\)
   2.4.4 Ratiometric measurements
2.5 Real-time imaging of mitosis .............................................................. 71
   2.5.1 Tissue preparation
   2.5.2 Imaging data acquisition, storage and analysis
2.6 Experiments to determine the effects of drugs \textit{in ovo} .................... 72
   2.6.1 Application of drugs \textit{in ovo}
   2.6.2 Preparation of retinal sections
   2.6.3 Data analysis
2.7 \textit{In vivo} dye labelling of retinal cells using a biolistic technique.... 75
   2.7.1 Coating biolistic particles with lipophilic dyes
   2.7.2 Particle delivery
   2.7.3 Imaging and data analysis
2.8 Dye-injections and determination of dye-coupling .......................... 77
   2.8.1 Dye filling using whole cell patch clamping
   2.8.2 Neurobiotin histochemistry
   2.8.3 Confocal imaging of filled cells
2.9 Immunocytochemistry ...................................................................... 79
   2.9.1 ChAT staining
   2.9.2 TuJ-1 staining
   2.9.3 Connexin staining
Chapter 3
Confocal imaging of spontaneous and neurotransmitter-evoked $[Ca^{2+}]_i$ signals in the VZ of the developing chick retina

3.1 Introduction .............................................................................................. 90
3.2 Methods .................................................................................................... 92
3.3 Results ....................................................................................................... 93
   3.3.1 Simultaneous recording of $[Ca^{2+}]_i$ signals and mitotic status by confocal microscopy .................................................. 93
   3.3.2 Interphase and mitotic cell populations in the VZ ................. 93
   3.3.3 Spontaneous $[Ca^{2+}]_i$ fluctuations in the VZ ....................... 94
   3.3.4 Cells throughout the depth of the retina undergo spontaneous $[Ca^{2+}]_i$ transients ............................................................... 95
   3.3.5 The proportion of VZ cells showing spontaneous $[Ca^{2+}]_i$ transients decreases between E4 and E6 .................................. 96
   3.3.6 Spontaneous $[Ca^{2+}]_i$ activity in the VZ is independent of action potentials ............................................................. 97
   3.3.7 Spontaneous $[Ca^{2+}]_i$ activity in the VZ results from endogenous release of neurotransmitter .............................................. 97
   3.3.8 Spontaneous $[Ca^{2+}]_i$ activity in the VZ is enhanced by eserine and iso-OMPA and reduced by apyrase ................... 98
   3.3.9 ACh produces changes in $[Ca^{2+}]_i$ in VZ cells in the early chick retina ................................................................. 100
   3.3.10 Pharmacology of the cholinergic response ................................ 100
   3.3.11 UTP produces changes in $[Ca^{2+}]_i$ in VZ cells in the early chick retina ................................................................. 102
   3.3.12 Pharmacology of the purinergic response ................................ 102
   3.3.13 GABA produces changes in $[Ca^{2+}]_i$ in VZ cells in the early chick retina ................................................................. 104
   3.3.14 Glutamate produces changes in $[Ca^{2+}]_i$ in VZ cells in the early chick retina ................................................................. 104
   3.3.15 Muscarinic and purinergic responses are mediated by the release of $[Ca^{2+}]_i$ from intracellular stores whilst GABA-
ergic and glutamatergic responses require the entry of Ca\(^{2+}\) from the extracellular space ....................................................... 105

3.3.16 The muscarinic and purinergic responses come from both mitotic and interphase cells ....................................................... 106

3.3.17 The GABAergic and glutamatergic responses come from a predominantly interphase population ...................................... 106

3.3.18 Developmental change in sensitivity to neurotransmitter stimulation ................................................................................... 107

3.3.19 VZ cells can respond to more than one neurotransmitter.... 108

3.3.20 Comparison of the relative effectiveness of purinergic and muscarinic agonists in evoking [Ca\(^{2+}\)] responses ............. 110

3.4 Discussion .................................................................................................. 111

3.4.1 Confocal Ca\(^{2+}\) imaging in the developing VZ ......................... 111

3.4.2 Endogenous release of neurotransmitter drives spontaneous [Ca\(^{2+}\)] activity in retinal VZ cells ............................................. 112

3.4.3 Muscarinic responses in embryonic chick retina .................... 113

3.4.4 Purinergic responses in embryonic chick retina .............. 114

3.4.5 GABAergic responses in embryonic chick retina ............ 118

3.4.6 Glutamatergic responses in embryonic chick retina .............. 118

3.4.7 Neurotransmitter responses change with age ......................... 119

3.4.8 The distribution of neurotransmitter responses within the interphase and mitotic cell populations .................................... 120

3.5 Further studies ........................................................................................... 123

Chapter 4

The effects of neurotransmitters on mitotic cells in the VZ of the developing chick retina

4.1 Introduction .................................................................................................. 147

4.2 Methods ........................................................................................................ 149

4.2.1 Real-time imaging of mitosis and metaphase spindle rotation

4.2.2 Application of drugs in ovo

4.2.3 Statistical analysis
4.3 Results

4.3.1 Mitosis can be followed in real time in whole mount retinae...

4.3.2 Time-lapse imaging of mitosis: the rotation of metaphase plates within the plane of the VZ

4.3.3 Muscarinic and purinergic, but not GABAergic or glutamatergic, receptors affect mitosis

4.3.4 Caffeine increases the time spent in prophase

4.3.5 BAPTA-AM blocks the purinergic and muscarinic [Ca\(^{2+}\)]\(_i\) responses, reduces spontaneous activity and prevents entry into metaphase

4.3.6 Purinergic, but not muscarinic, stimulation partially rescues cells from the effects of caffeine

4.3.7 Muscarinic and purinergic, but not GABAergic or glutamatergic, receptors affect eye growth

4.3.8 Muscarinic and purinergic, but not GABAergic or glutamatergic, receptors affect the number of cells in mitosis in the VZ

4.3.9 The plane of cell division is unaffected by neurotransmitter receptor activation

4.3.10 Mitosis is faster when the RPE is present

4.3.11 Apyrase slows the rate of mitosis when the RPE is present

4.4 Discussion

4.4.1 Rotations of the metaphase plate

4.4.2 Muscarinic and purinergic, but not ionotropic glutamatergic or GABAergic, receptors regulate the cell cycle

4.4.3 [Ca\(^{2+}\)]\(_i\) and the cell cycle

4.4.4 Receptors can use the same downstream effectors to cause different effects

4.4.5 Symmetric and asymmetric division

4.4.6 Developmental consequences of purinergic and muscarinic regulation of proliferation

4.4.7 Potential RPE-mediated purinergic regulation of mitosis

4.5 Further studies
Chapter 5
Confocal imaging of interkinetic nuclear migration using a biolistic labelling technique

5.1 Introduction ................................................................. 204
5.2 Methods ........................................................................ 206
5.3 Results ........................................................................... 207
5.3.1 Labelling retinal cells using the ‘DiOlistic’ technique .......... 207
5.3.2 The morphology of migrating retinal cells as identified by biolistic labelling .............................................. 208
5.3.3 Retraction of processes in preparation of mitosis .......... 210
5.3.4 PCs undergoing INM move in a saltatory manner ......... 210
5.3.5 Speed of nuclear translocation during INM ............... 211
5.3.6 [Ca\textsuperscript{2+}] \textsubscript{i} transients are associated with movement .......... 212
5.3.7 The effects of neurotransmitters on INM in the E5 chick retina ......................................................... 213

5.4 Discussion ....................................................................... 216
5.4.1 The ‘DiOlistic’ technique and INM in the chick retina .... 216
5.4.2 [Ca\textsuperscript{2+}] \textsubscript{i} transients are temporally correlated with INM .... 219

5.5 Further studies .................................................................. 221

Chapter 6
Gap junctional communication within and between the RPE and the VZ during early development of the chick retina

6.1 Introduction ..................................................................... 238
6.2 Methods .......................................................................... 241
6.3 Results ............................................................................. 243
6.3.1 Spontaneous [Ca\textsuperscript{2+}] \textsubscript{i} transients in the RPE ......................... 243
6.3.2 Spontaneous co-ordinated [Ca\textsuperscript{2+}] \textsubscript{i} transients in the VZ in the absence of the RPE ................................................. 244
6.3.3 Spontaneous co-ordinated and isolated [Ca\textsuperscript{2+}] \textsubscript{i} transients in the VZ in the presence of the RPE ................................. 245
6.3.4 Transmission of spontaneous [Ca\textsuperscript{2+}] \textsubscript{i} transients between the RPE and the VZ ............................................. 246
6.3.5. The embryonic RPE responds to muscarinic and purinergic stimulation with a change in $[Ca^{2+}]_i$. ............................................ 248

6.3.6. Cells in the neural retina can be dye-filled using whole cell patch clamping. ................................................................. 249

6.3.7. Dye-coupling results from diffusion of Neurobiotin through gap junctions. ......................................................................... 250

6.3.8. In the E5 retina clusters of coupled cells largely consist of PCs 250

6.3.9. RPE cells are dye-coupled. ........................................................ 252

6.3.10 Connexin staining is found at the interface between the RPE and the VZ.............................................................. 253

6.3.11 Dye coupling occurs between cells of the RPE and the neural retina .............................................................. 253

6.4 Discussion

6.4.1. Dye coupling between cells in the neural retina..................... 255

6.4.2. Potential roles for coupling between neural retinal PCs............ 258

6.4.3. Dye coupling within the RPE and between the RPE and the neural retina.............................................................. 259

6.4.4. Potential roles for coupling between the RPE and the neural retina.............................................................. 260

6.4.5. Co-ordinated $[Ca^{2+}]_i$ activity in the RPE and neural retina..... 261

6.4.6. The RPE response to neurotransmitters................................. 263

6.5 Further studies................................................................................. 264

Chapter 7 Conclusions.................................................................... 281

Publications ....................................................................................... 284

References ......................................................................................... 286
**List of Figures**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Anatomy of the mature chick retina</td>
<td>59</td>
</tr>
<tr>
<td>1.2</td>
<td>Mitosis</td>
<td>60</td>
</tr>
<tr>
<td>1.3</td>
<td>Interkinetic nuclear migration</td>
<td>61</td>
</tr>
<tr>
<td>1.4</td>
<td>Cell birth in the chick retina</td>
<td>62</td>
</tr>
<tr>
<td>1.5</td>
<td>Symmetric and asymmetric cell division</td>
<td>63</td>
</tr>
<tr>
<td>1.6</td>
<td>Neurotransmitter receptor expression in the developing retina</td>
<td>64</td>
</tr>
<tr>
<td>2.1</td>
<td>The perfusion system</td>
<td>83</td>
</tr>
<tr>
<td>2.2</td>
<td>Ratiometric measurements</td>
<td>84</td>
</tr>
<tr>
<td>2.3</td>
<td>Measurement of the time spent in metaphase during mitosis</td>
<td>85</td>
</tr>
<tr>
<td>2.4</td>
<td>Measurement of eye diameter and the angle of division</td>
<td>86</td>
</tr>
<tr>
<td>2.5</td>
<td>'DiOlistic' labelling of the retina</td>
<td>87</td>
</tr>
<tr>
<td>2.6</td>
<td>Reconstruction of 3D time-series</td>
<td>88</td>
</tr>
<tr>
<td>2.7</td>
<td>Measurement of interkinetic nuclear migration</td>
<td>89</td>
</tr>
<tr>
<td>3.1</td>
<td>Dual-labelling of VZ cells with Fluo 4-AM and Hoechst 33342</td>
<td>124</td>
</tr>
<tr>
<td>3.2</td>
<td>Spontaneous [Ca$^{2+}$] transients in E6 VZ cells</td>
<td>125</td>
</tr>
<tr>
<td>3.3</td>
<td>Spontaneous [Ca$^{2+}$] transients occur throughout the depth of the neural retina</td>
<td>126</td>
</tr>
<tr>
<td>3.4</td>
<td>Propagation of [Ca$^{2+}$] transients</td>
<td>127</td>
</tr>
<tr>
<td>3.5</td>
<td>The Na$^+$ channel blocker TTX does not reduce the frequency of spontaneous events</td>
<td>128</td>
</tr>
<tr>
<td>3.6</td>
<td>Spontaneous [Ca$^{2+}$] activity results from endogenous activation of neurotransmitter receptors</td>
<td>129</td>
</tr>
<tr>
<td>3.7</td>
<td>ChAT immunoreactivity is present in the early embryonic retina</td>
<td>130</td>
</tr>
<tr>
<td>3.8</td>
<td>Spontaneous [Ca$^{2+}$] activity can be increased by preventing the breakdown of endogenous ACh</td>
<td>131</td>
</tr>
<tr>
<td>3.9</td>
<td>Spontaneous activity can be decreased by increasing the breakdown of endogenous ATP</td>
<td>132</td>
</tr>
</tbody>
</table>
Figure 3.10. VZ cells in early embryonic retina respond to muscarinic stimulation..................................................................................... 133

Figure 3.11. The response to CCh is mediated by muscarinic receptors........................................... 134

Figure 3.12. The response to CCh is blocked by atropine and pirenzipine............................................. 135

Figure 3.13. Pharmacology of the cholinergic response................................................................. 136

Figure 3.14. VZ cells in early embryonic retina respond to purinergic stimulation.................................................. 137

Figure 3.15. Pharmacology of the purinergic response: agonists................................................ 138

Figure 3.16. Pharmacology of the purinergic response: antagonists........................................... 139

Figure 3.17. VZ cells in early embryonic retina respond to GABAergic and glutamatergic stimulation.............................................................................. 140

Figure 3.18. The muscarinic and purinergic responses are mediated by the release of Ca$$^{2+}$$ from intracellular stores whilst GABAergic and glutamatergic responses result from Ca$$^{2+}$$ entry through VOCCs............................................................................ 141

Figure 3.19. Mitotic and interphase VZ cell populations respond to different agonists.......................... 142

Figure 3.20. Responses to GABA arise from NDCs...................................................................... 143

Figure 3.21. Developmental changes in sensitivity to neurotransmitters.......................................... 144

Figure 3.22. Cells in the VZ can respond to more than one neurotransmitter.................................. 145

Figure 3.23. The response to UTP and CCh differs in amplitude and Frequency................................................. 146

Figure 4.1. Real-time confocal imaging of mitosis........................................................................ 185

Figure 4.2. Time lapse series of mitosis.................................................................................. 186

Figure 4.3. Rotations of the mitotic apparatus within the plane of the VZ........................................ 187

Figure 4.4. Paths of metaphase rod rotation during mitosis in the VZ........................................... 188

Figure 4.5. Rose diagrams of the orientations of chromatin during metaphase and anaphase.................................................. 189

Figure 4.6. Time spent in metaphase is affected by muscarinic and purinergic, but not by GABAergic or glutamatergic, stimulation 190

Figure 4.7. Time spent in metaphase is affected by muscarinic but not purinergic inhibition................................................. 191
Figure 4.8. Eye size is affected by muscarinic and purinergic, but not GABAergic or glutamatergic, receptor activation and blockade in ovo. ................................. 192

Figure 4.9. Changes in eye size following muscarinic stimulation or inhibition ................................................................................. 193

Figure 4.10. Cell density and cell death are not affected by muscarinic, purinergic, GABAergic or glutamatergic stimulation in ovo. 194

Figure 4.11. Mitosis is affected by muscarinic and purinergic, but not GABAergic or glutamatergic, receptor activation and blockade in ovo ......................................................... 195

Figure 4.12. Horizontal and vertical PC divisions .................................................. 196

Figure 4.13. Orientations of the metaphase rod in dividing PCs ........................................ 197

Figure 4.14. The effect of purinergic drugs on the plane of cleavage ............... 198

Figure 4.15. The effect of muscarinic drugs on the plane of cleavage ............... 199

Figure 4.16. The effect of GABAergic drugs on the plane of cleavage ........... 200

Figure 4.17. The effect of glutamatergic drugs on the plane of cleavage ...... 201

Figure 4.18. Mitosis in the VZ is speeded up in the presence of the RPE .......... 202

Figure 4.19. Release of a purinergic agonist by the RPE may regulate mitosis in the VZ ................................................................. 203

Figure 5.1. INM during the PC proliferative cycle ........................................ 222

Figure 5.2. ‘DiOlistic’ labelling of retinal cells ............................................. 223

Figure 5.3. A combination of DiI and DiO allows neighbouring cells to be labelled different colours .............................................. 224

Figure 5.4. Biolistic labelling does not disrupt dynamic and physiological changes in retinal cells ........................................ 225

Figure 5.5. INM in PCs moving towards the VZ ......................................... 226

Figure 5.6. INM in cells moving towards the GCL ....................................... 227

Figure 5.7. Process retraction in PC and NDCs ............................................. 228

Figure 5.8. INM is saltatory in PCs moving towards the VZ ....................... 229

Figure 5.9. INM at higher image acquisition frequencies ............................ 230

Figure 5.10. Speed of cell movement ......................................................... 231
Figure 5.11. [Ca$$^{2+}$$], transients are temporally correlated with cell movement....................................................................................... 232
Figure 5.12. Relationship between [Ca$$^{2+}$$], transients and PC INM........... 233
Figure 5.13. UTP evokes [Ca$$^{2+}$$], responses in cells throughout the depth of the retina...................................................................................... 234
Figure 5.14. CCh evokes [Ca$$^{2+}$$], responses in cells throughout the depth of the retina............................................................................. 235
Figure 5.15. The effects of purinergic receptor activation/blockade on INM 236
Figure 5.16. The effects of cholinergic receptor activation/blockade on INM 237

Figure 6.1. Spontaneous [Ca$$^{2+}$$], waves in the E5 RPE....................... 266
Figure 6.2. Spontaneous [Ca$$^{2+}$$], transients in the E5 RPE.................. 267
Figure 6.3. Mitotic cells are rarely involved in [Ca$$^{2+}$$], waves in the VZ..... 268
Figure 6.4. Spontaneous [Ca$$^{2+}$$], waves in the E5 VZ ......................... 269
Figure 6.5. Co-ordinated spontaneous [Ca$$^{2+}$$], activity involves cells throughout the depth of the neural retina................................. 270
Figure 6.6. Spontaneous [Ca$$^{2+}$$], activity in the VZ is higher when the RPE is in place................................................................. 271
Figure 6.7. Propagation of a spontaneous [Ca$$^{2+}$$], wave from the neural retina to the RPE............................................................... 272
Figure 6.8. [Ca$$^{2+}$$], activity propagates from the RPE to the VZ........... 273
Figure 6.9. The E5 RPE responds to purinergic and muscarinic stimulation. 274
Figure 6.10. Dye coupling in the neural retina.......................................... 275
Figure 6.11. Dye coupling of cells in the E5 neural retina......................... 276
Figure 6.12. Mitotic cells may not be coupled to other PCs..................... 277
Figure 6.13. RPE cells are coupled by gap-junctions................................. 278
Figure 6.14. Cx43 staining in E5 retina....................................................... 279
Figure 6.15. RPE cells are coupled with the neural retina....................... 280
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Chapter 1

Introduction

The chick retina consists of approximately two hundred million cells, nearly all of which are produced within the 10-day period following the formation of the optic cup. This corresponds to the production of about 250 cells a second. All these cells must be produced in the correct number, adopt the correct fate, migrate to their final destination and form synaptic connections with the appropriate partners. To ensure the proper function of the adult retina, these processes must be highly regulated.

This thesis investigates some of the potential roles of neurotransmitters in regulating development prior to the formation of synapses. This chapter provides a brief introduction to retinal development and some of the factors thought to be involved in its regulation. Chapter 2 describes the methods employed in the experiments presented in this thesis. Chapter 3 describes spontaneous and neurotransmitter-evoked $[Ca^{2+}]_i$ activity occurring in the early embryonic chick retinal VZ. Chapter 4 examines the effects of neurotransmitters and the RPE on mitosis. Chapter 5 describes the development of techniques to enable the observation of PC migration and associated $Ca^{2+}$ signals. Chapter 6 reports an investigation of the gap junction-mediated coupling between cells in the neural retina and between cells of the RPE and the neural retina.

1.1 Organisation of the Retina

The mature chick neural retina consists of three layers of cell bodies. The outermost layer of these, the outer nuclear layer (ONL), lies juxtaposed to the RPE at the back of the eye capsule. This is separated from the next layer of cell bodies, the inner nuclear layer, (INL) by the neuropil of the outer plexiform layer (OPL). The INL is then separated from the ganglion cell layer (GCL) and fibre layers (FL) by the dendrites and axon terminals of the inner plexiform layer (IPL) (Figure 1.1).

There are seven cell types in the chick retina, of which six are neuronal: ganglion cells, amacrine cells, horizontal cells, bipolar cells and photoreceptors (rods and cones). In addition, glial cells are present in large numbers. The mammalian retina contains
astrocytes and radial glia called Müller cells. However, as in other lower vertebrates, the Müller cell is the only glial cell type found in the chick retina. Müller cells extend processes to either side of the retina and their cell bodies lie in the INL. The photoreceptors lie in the region immediately adjacent to the RPE. Their cell bodies are located in the ONL and their terminals form synapses with the dendrites of bipolar and horizontal cells in the OPL. The cell bodies of the bipolar, amacrine and horizontal cells lie in the INL. Bipolar cell axon terminals and amacrine cell dendrites are located in the IPL along with the dendrites of retinal ganglion cells (RGCs), the cell bodies of which are located in the GCL. The axons of RGCs run in the FL to the optic nerve and terminate in the optic tectum of the brain. Some amacrine cell bodies are found in the GCL and are termed “displaced” amacrine cells. Likewise, “displaced” RGC bodies are sometimes seen in the INL and “displaced” horizontal and bipolar cells can be found in the ONL.

The basic circuitry of the retina arises from the organisation of these neuronal types into vertical and lateral pathways. In the vertical pathway, photoreceptors synapse with bipolar cells, which in turn connect with the RGCs and so with the brain via the optic nerve. A single photoreceptor may connect with several bipolar cells, each of which contacts one or many RGCs. In addition to this ‘direct’ line, the horizontal and amacrine cells form lateral pathways. Horizontal cells receive inputs from many photoreceptors and in turn synapse back on to them, as well as on to bipolar cells. Similarly, amacrine cells receiving inputs from bipolar cells send synapses back to them and on to the RGCs. Thus, the horizontal cells and amacrine cells modify the transfer of information through the retina at the level of the OPL and IPL, respectively. Receptive fields are defined as the specific area on the retinal surface that affects, upon illumination, the signalling of an individual neuron.

1.2 Information processing in the retina

The neural response to light begins with the activation of a biochemical cascade within the outer segments of rods and cones. Rod photoreceptors mediate vision at low light intensities, whilst cones operate in bright light and mediate colour vision. In the chick retina, cones vastly outnumber rods (Walls, 1942) and contain one of four different visual pigments; red, blue, green and UV (for review, see Okano et al., 1995). In
vertebrates, both photoreceptor types respond to light with a hyperpolarizing receptor potential. The cell membrane in the outer segment of the receptor is highly permeable to cations in the dark. As a result, there is a constant influx of sodium (Na\(^+\)) into the outer segment known as the ‘dark current’. This is balanced electrically by an outward flux of potassium (K\(^+\)) in the inner segments and the electrochemical gradients are maintained by the action of the Na\(^+\)/K\(^+\)-ATPase. The dark current causes the cell to be depolarised, relative to the predicted resting membrane potential, and causes a tonic release of the neurotransmitter glutamate from the receptor synapse on to postsynaptic cells. Following the absorption of light by the photopigments (located in the outer segment) the Na\(^+\) conductance in the outer segment decreases and, consequently, so does the dark current. As a result, the membrane potential hyperpolarizes, leading to a reduction in the tonic level of glutamate release.

Illumination reduces the dark current via a biochemical cascade. The rod visual pigment, rhodopsin, has been intensively studied. It is a seven-pass transmembrane protein, homologous to other receptors that activate G-proteins, and is covalently attached to the chromophore 11-cis-retinal. Upon absorption of a single photon, the chromophore isomerises to all-trans retinal causing a conformational change in the rhodopsin molecule. The light-activated photopigment induces the activation of a G-protein, which in turn disinhibits a phosphodiesterase causing the hydrolysis of cGMP. In the dark, cGMP holds the cation channels in the outer segment membrane open. Upon hydrolysis of cGMP the channels close, shutting off the influx of cations, resulting in membrane hyperpolarization.

Bipolar and horizontal cells receive convergent inputs from several photoreceptors and respond to the reduction in glutamate release caused by light with sustained, graded potentials. Bipolar cells may be ON-centre (and depolarise in response to illumination of their receptive field centre) or OFF-centre (and hyperpolarize when light falls on the receptive field centre). The axon terminals of bipolar cells release glutamate on to the dendrites of RGCs and amacrine cells in the IPL. RGCs respond to light with either an increase or a decrease in the frequency of the action potentials they send to the brain, depending on whether they are driven by ON or OFF bipolar cells. Horizontal cells hyperpolarize in response to light, leading to a reduction in the release of the inhibitory neurotransmitter \(\gamma\)-amino butyric acid (GABA) onto the cones and bipolar cells with
which they synapse. Lateral interactions in the OPL mediated by horizontal cells provide bipolar cells with centre-surround receptive fields and, via this mechanism, form the basis for the detection of contrast. In the IPL, amacrine cells provide change-dependent lateral inhibition of RGCs, tuning them for the detection of motion.

1.3 The chick retina as a model for developmental studies

The chick retina is an ideal model for the investigation of central nervous system (CNS) development because: i) like other regions of the CNS, it derives from the neural tube, ii) the timetable for chick retinal development is well documented, iii) the adult structure has been intensively studied, iv) the development of the chick in the egg permits easy access to the retina at any time, v) the retina’s laminated architecture, flat and transparent nature ideally suit it to imaging experiments and vi) dividing cells are located within a narrow proliferative zone, enabling them to be imaged in a single focal plane.

The next section describes the development of the eye, including the proliferative cell cycle and programmed cell death, which together determine cell numbers in the adult retina.

1.4 Development of the Retina

1.4.1 Early development of the eye

During vertebrate embryogenesis, the nervous system is induced to form from a sheet of ectoderm by the underlying dorsal mesoderm of the gastrula. The nervous system starts as a sheet of neuroectodermal cells, the neural plate. The edges of this sheet thicken and move upwards into neural folds that fuse at the midline to form a hollow neural tube. The retina is formed through the evagination of the lateral walls of the developing neural tube, in the region destined to become the midbrain, which gives rise to the optic vesicle. In the chick embryo, this process begins at around 30 hours (h) post-fertilization. The base of each vesicle constricts until, by 48h, its connection to the brain is reduced to a narrow stalk. The primordial optic vesicles subsequently invaginate to form a two-layered structure, the optic cup. The inner layer of this structure will develop
to produce the neural retina whilst the outer layer of epithelial cells forms the RPE. Initially, both layers are only one cell thick, but the inner layer consists of neuroblasts that divide repeatedly to produce the neurons and glia of the neural retina. Within the neural retina, mitosis is confined to the layer of cells immediately adjacent to the RPE, a region called the VZ.

Retinal development occurs in two overlapping phases: proliferation and differentiation (Young, 1983). During the first phase, PCs undergo cycles of division to increase the number of cells in the progenitor pool. In the second stage, cell division gives rise to newly born neurons and glia that differentiate into the cell types of the adult tissue and migrate to their final destination, before forming synaptic connections with other retinal neurons. RGCs and amacrine cell number is regulated by cell death (which reduces the numbers by as much as 50% in some species—see section 1.4.4). However for other cell types, such as photoreceptors, which are not extensively regulated by apoptosis (Cook et al., 1998), neurogenesis must be closely controlled in order to produce the correct number of each type.

1.4.2 The cell cycle

The cell cycle can be divided into 4 sequential steps: G\textsubscript{1}, S-phase, G\textsubscript{2} and mitosis (Figure 1.2a). G\textsubscript{1}, S-phase and G\textsubscript{2}, collectively, are called interphase. DNA duplication occurs during S-phase. The two gap phases (G\textsubscript{1} and G\textsubscript{2}) serve to provide time for the cell to monitor the internal and external environment, and to prepare for DNA replication and cell division, respectively. Mitosis itself consists of several stages (see Figure 1.2b for detail). Upon entering prophase, the chromosomes condense and each chromosome pair attaches to the mitotic spindle via its centromere. The duplicate pairs then line up across the equatorial plane of the cell in metaphase. The chromosomes form a dense band across the middle of the cell, referred to as the metaphase plate. Upon entering anaphase, the chromosome pairs are pulled to opposite poles of the dividing cell by the action of the spindle. During telophase the chromosomes reach the poles and de-condense prior to the formation of new nuclear membranes and disassembly of the mitotic apparatus. Cytokinesis, the constriction of cytoplasmic material round the equatorial plane, may begin before the nuclear material enters telophase and is complete when two new daughter cells are produced. Many terminally
differentiated cells, such as neurons, do not divide further and assume a quiescent state called the $G_0$ phase. The cell cycle of chick retinal cells typically occupies a 6-16h period (Morris and Cowan, 1995) with the length increasing during development.

In the developing retina, and throughout most CNS tissue, the nuclei of PCs migrate up and down throughout the depth of the neural tissue, in a process termed 'interkinetic nuclear migration' (INM) (Sauer, 1935; Sidman et al., 1959; Sidman, 1961) (see Figure 1.3a for detail). Mitosis only occurs in the VZ (Figure 1.3b) and so this region may be considered as 'permissive' for mitosis. Each new retinal PC extends a process to the vitreal surface and also retains a connection with the ventricular surface (Figure 1.3c). The nucleus moves along the process, from the VZ to the prospective GCL during $G_1$, before duplicating its DNA (S-Phase) and returning to the VZ during $G_2$. Upon reaching the VZ, the vitreal process is withdrawn, the cytoplasm is contracted into a sphere and the cell enters mitosis. The resulting daughter cells separate, extend vitreal processes and start the cycle again.

1.4.3 Cell birth during retinal development

Retinal cells originate from a population of pluripotential PCs. A single PC will produce a clone of mixed cell types (Turner and Cepko, 1987; Holt et al., 1988). The process of proliferation overlaps with the onset of differentiation. In the chick, neuronal cell birth begins at embryonic day (E) 2, continues until at least E8 in the central retina, and is largely complete by E12 (Prada et al., 1991). Cell determination in the retina follows a temporal order, although there is extensive overlap in the time at which different cell types are born (Figure 1.4). Cells that are born and differentiate early include the majority of RGCs. Horizontal cells, cones and some types of amacrine cells also start to appear during the period of the production of RGCs, while later born cells include bipolar cells, rods and Müller cells (Sidman, 1961; Prada et al., 1991). The precise order varies according to species and consistent birth dates for individual cell types have proved hard to determine in the chick.

Chick RGCs start to be born at E2 and ~40% of RGCs have undergone their final mitosis by E3 (Snow and Robson, 1994). The GCL becomes a distinct layer around E7 although synapses are not seen until E12 (Hughes and LaVelle, 1974). Some cells
express RGC markers immediately after their last mitotic division, before migrating to their definitive laminar position (Waid and McLoon, 1995). However, it remains to be determined whether the expression of such markers reflects an irreversible commitment of those PCs to a RGC fate, or is a transient phenomenon that becomes permanent only upon reaching the GCL. Prada et al. (1991) have shown that amacrine and horizontal cells start to leave the cell cycle from E4 (3 and 2%, respectively, are post-mitotic at this stage), followed by photoreceptors (7% are postmitotic at E5). Bipolar cells are the last neuronal cells to leave the cycle, starting to exit at E5. RGCs attain the 50% postmitotic level by E5; horizontal, photoreceptors and amacrine cells do so during E6 and bipolar cells during E7 (Prada et al., 1991). Müller cell genesis has generally been considered to take place after the generation of retinal neurons is complete (Prada et al., 1991; Linser et al., 1997, but see section 1.5). The RPE forms with the initial neural retina and starts to differentiate at ~E3 in the chick.

A spectrum of developmental stages coexists in the retina at any given embryonic stage. In the chick retina, both proliferation and differentiation occur in a centrifugal manner (Kahn, 1974). Autoradiographic studies have shown that neurogenesis starts in the central region of the retina (Prada et al., 1991). A gradient of neurogenesis then spreads out towards the periphery, so that at any given stage the centre of the retina is more developmentally advanced with respect to the edge. In addition to this centrifugal gradient, there are distinct temporal-to-nasal and dorsal-to-ventral gradients. Many more post-mitotic cells are found in the temporal, compared with the nasal, areas of the retina between E6 and E8, in both dorsal and ventral regions. Similarly, dorsal sectors are consistently more advanced through neurogenesis than the corresponding ventral ones (Prada et al., 1991).

1.4.4 Cell death during retinal development

Programmed cell death is a widespread phenomenon and is essential for the normal development of the nervous system. The precise developmental roles of this process are not well understood but they appear to include the regulation of final cell number and the elimination of aberrant connections during synaptogenesis. Cell death is often characterized morphologically by condensation of nuclear chromatin and fragmentation of DNA, cytoplasmic condensation and membrane breakdown. It is also accompanied
by a progressive increase in \([\text{Ca}^{2+}]_i\) (for review, see Cellerino et al., 2000). Cell death can be induced by a multitude of stimuli including changes in electrical activity and the removal of trophic support, and the final common pathway involves the stimulation of cytokine receptors and caspase activation.

Gonzalez-Hoyuela et al., (2001) have shown that RGCs appear to regulate their own numbers by inhibiting the generation of new RGCs and by killing incoming migratory neurons via a process involving nerve growth factor (NGF). Selective ablation of RGCs in the chick retina resulted in a repopulation of RGCs and a large decrease in cell death. Application of exogenous NGF was able to reverse the effects of RGC ablation on the levels of cell death. Given that the only source of NGF in the retina is the RGCs themselves, it suggests that these cells may regulate their own numbers via the secretion of NGF. NGF-mediated cell death occurs during RGC differentiation and affects post-mitotic cells migrating from the VZ to the prospective GCL. Exogenous application of brain-derived neurotrophic factor (BDNF) inhibits the cell death that occurs during this period (Frada et al., 1997) and thus a balance appears to exist between the trophic actions of BDNF and the apoptotic properties of NGF.

Programmed cell death is not a universal process among the cell types of the chick retina. Cook et al. (1998) examined cell death in retinal sections at different stages of development. TUNEL-positive cells and pyknotic nuclei (markers for apoptotic cell death) were first detected in the GCL around E8, peaking at E10. Similarly, these markers were observed from E8 in the INL, with the levels peaking around E11 but were largely absent at E14. However, dying cells were almost never seen in the ONL, suggesting that programmed cell death may not be the primary mechanism regulating photoreceptor numbers.

**1.4.5 Symmetrical and asymmetrical division of progenitor cells**

Several investigators have speculated that the orientation of the mitotic cleavage plane of precursors undergoing terminal division may act as a means to regulate the production of neurons (Langman et al., 1966; Martin, 1967). Three modes of division have been proposed: 1) symmetric non-terminating, in which division of a PC results in two identical progenitor daughter cells 2) asymmetric, producing one PC and newly
differentiated cell (NDC) and 3) symmetric terminating, whereby both daughter cells adopt a postmitotic state (Takahashi et al., 1994; Caviness et al., 1995; Figure 1.5). Symmetrical non-terminating divisions expand the progenitor pool as is required in the initial stages of development. At the onset of neurogenesis, some cells switch to asymmetric division. Neurogenesis occurs in overlapping waves, with proliferation continuing throughout much of this period (see above). Thus, as neurogenesis progresses, there is a shift from the symmetric to the asymmetric mode of PC division (Chenn and McConnell, 1995; Caviness et al., 1995). Towards the end of neurogenesis symmetric terminating divisions dominate, leading to the eventual depletion of the progenitor pool and cessation of neurogenesis.

Recent investigations have examined the mechanisms regulating the different behaviours of daughter cells following either symmetric or asymmetric divisions. Some of the first direct evidence that these modes of division could produce daughter cells of differing fate came from time-lapse imaging studies of the cleavage plane and daughter cell behaviour in the developing ferret telencephalon (Chenn and McConnell, 1995). Chenn and McConnell further proposed that the orientation of the cleavage plane, with respect to the epithelium, influenced the resulting division. Thus, an asymmetric division is defined as one in which division occurs perpendicular to the plane of the epithelium (see Figure 1.5a). This results in one daughter cell that retains progenitor-like morphology and behaviour and another that migrates rapidly out of the VZ, and adopts the morphology of an immature neuron. Conversely, symmetric divisions occur within the plane of the developing epithelium and give rise to two daughter cells that move away from the VZ at a similar rate, remain in close proximity to one another, and are progenitor-like in appearance.

The timing of commitment to a symmetric or an asymmetric division, and the control mechanisms involved, remain unclear. Time-lapse confocal studies in the developing rat cortex (Adams, 1996) show that the metaphase plates of dividing cells undergo multiple rotations prior to entering anaphase (Figure 1.5b). These rotations are restricted to a particular plane. In the case of symmetrical divisions the metaphase plates rotated so that the spindle remained solely within the plane of the VZ. 'Flipping' of the metaphase plate to that consistent with asymmetric division was seldom seen. Other cells were observed that appeared to have an orientation that was consistent with an asymmetric
division (i.e. the mitotic spindle is perpendicular to the plane of the VZ), and these also
rotated within their respective plane. Thus, the decision of whether to divide
horizontally or vertically, with respect to the VZ, appears to be determined prior to entry
into metaphase, although a cell may then spend some considerable time searching for
the appropriate orientation within the plane of division (Adams, 1996).

Asymmetric division provides a mechanism for the asymmetric inheritance of cell fate
determinants (for reviews see Jan and Jan, 2001; Knoblich, 2001). In Drosophila, the
determinant proteins Numb and Prospero become localized to the basal side of
dividing neuroblasts. The polar segregation of these factors is tightly coupled to the
orientation of the spindle. Evidence from budding yeast suggests that the spindle
undergoes a series of see saw-like movements until the spindle microtubules find
binding sites within the cell cortex (for review, see Jan and Jan, 2001). An asymmetric
division results in such cell fate determinants being inherited by the basal daughter cell
alone. Many of the factors identified in Drosophila have mammalian and avian
homologues, suggesting conserved roles for these molecules in the developing nervous
system. Notch1, the mammalian homologue of Drosophila Notch, is distributed
asymmetrically in PCs in the developing cerebral cortex of the ferret (Chenn and
McConnell, 1995). Notch immunoreactivity is localized to the basal region of dividing
cells. An asymmetric division would result in only the basal daughter inheriting the
protein, whilst a symmetric division should allow both daughters to inherit equally.
Another cell fate determinant, m-Numb, is segregated asymmetrically in dividing
mammalian cells. It is bound to the apical cell membrane in murine cortical PCs (Zhong
et al., 1996) and is apically located in both mitotic and interphase cells in the neonatal
retina (Cayouette et al., 2001) of the rat. Together with the findings of Chenn and
McConnell, these observations suggest that in an asymmetric division the m-Numb
would be inherited by the apical daughter cell, which would then remain proliferative.
This is perhaps surprising, given the accepted view of Notch in lateral inhibition and its
interactions with Numb in the determination of cell fate (for review, see Lewis, 1998).
The presence of Notch signalling is usually required for maintaining the proliferative
state, and Numb acts to reduce Notch signalling. However, it is now becoming clear that
Notch signalling is far more complex than first thought and that it may have diverse
roles in development, including promotion of differentiation (for review, see Wang and
Barres, 2000).
Other molecules may play important roles in the different modes of division. Electron micrographs reveal the apical processes of cortical PCs contain junctional complexes of proteins that contact the basal lamina (Aaku-Saraste et al., 1996). During mitosis, the junctional complex is retained. In symmetrical divisions the junctional apparatus is divided between the two daughters but only the apical cell retains the complex in an asymmetrical division (Hinds and Ruffet, 1971). Inheritance of the junctional complex may be an important factor in permitting a cell to continue to proliferate. Gap junctions have been found between the neural retina and the RPE (Hayes, 1976). If these were present between dividing PCs and the RPE they would be expected to be inherited by the apical daughter cell alone.

Different patterns of cell division act not only to regulate the production of PCs and differentiated cells but may also co-ordinate the spatial organization of adult structures. The ‘radial unit hypothesis’ (Rakic 1988) has been proposed to explain how clonally related neurons form radially aligned arrays within the cortex. A series of asymmetric divisions of a single PC leads to the sequential generation of siblings (for review, see Kornack, 2000). These then migrate along a common radial path and take up sequential ‘inside-out’ positions in the cortex. Retroviral lineage tracing in monkeys (Kornack and Rakic, 1995) and rodents (Mione et al., 1997) have revealed other arrays of clonally related neurons that are aligned horizontally within a single cortical lamina. This suggests they are generated at a similar time to one another and migrate in synchrony to the appropriate cortical layer. It is proposed that these are generated from a common ancestor cell by a series of symmetric divisions, giving rise to multiple PCs that simultaneously generate ‘cousins’ (Kornack and Rakic, 1995). The mechanisms that could co-ordinate the synchronised proliferation and migration of siblings are poorly understood.

1.4.6 Are radial glial cells progenitor cells?

The descriptions of PCs giving rise to all neuronal and non-neuronal cell types in the retina, and in the developing brain, have been in place for over a century. However, recent evidence has called into question the distinction between PCs and radial glia in the developing cortex, and perhaps the radial glia of the retina, Müller cells.
The traditional view of the role of radial glia was derived from electron microscopical studies in the developing telencephalon (Rakic, 1972). This work strongly suggested that radial glia provide a transient scaffold for, and impose radial constraints on, migrating newborn neurons. Until recently, it has been assumed that during neurogenesis the embryonic VZ is composed of neuroepithelial cells that give rise to distinct populations of neurons and glia, including radial glial cells. Despite the similarities in the morphology of PCs and radial glia, it was thought to be impossible for radial glial cells to divide. However, there is now strong evidence to suggest that radial glial cells can re-enter the cell cycle. They are immunopositive for nestin, an intermediate filament protein expressed in CNS PCs (Chanas-Sacre et al., 2000), and their nuclei can undergo INM (Noctor et al., 2002). In vivo retroviral labelling techniques have demonstrated that clones of cells arising from the division of a PC contain neurons and many glia but only one radial glial cell (Gray and Sanes, 1992). More recently, Malatesa et al. (2000) have directly examined the progeny of radial glia in a transgenic mouse line that expresses green fluorescent protein (GFP) under the control of a glial fibrillary acidic protein (GFAP) promoter. Sorting and culturing of GFP* cells demonstrated that firstly, these cells have a radial glia identity (as identified by immunohistochemical characterisation) and secondly, that they were able to give rise to new neurons. Using intraventricular injection of retrovirus to gain expression of GFP, Noctor et al. (2001) have shown that 24h after injection only radial glial cells are labelled, but that at later times the radial clones also contain neurons located along the radial fibre. BrdU staining showed that in any given clone, the radial glial cell was the only mitotically active cell. Using timelapse microscopy the same group show that radial glia undergo INM and divide asymmetrically, whilst apparently maintaining their radial processes, and giving rise to mitotic radial glia and postmitotic neurons (Noctor et al., 2002). Thus, recent evidence indicates that not only are radial glia capable of self-renewal, but also of the generation of neurons during early corticogenesis and, at later stages, of astrocytes.

The distinction between progenitor and radial glial cells remains unclear. For example, at what point do PCs, which are still thought to be responsible for the initial expansion of the progenitor pool, differentiate into radial glia? To resolve this question, specific
markers that distinguish differentiated radial glial cells from the progenitor population will need to be developed.

As indicated above the morphology of PCs, both in the cortex and the retina, and radial glial cells and Müller cells are remarkably similar. Can Müller cells in the retina perform a role similar, in terms of proliferation and neurogenesis, to that of radial glia in the cortex? A number of studies have shown that differentiated Müller cells appear relatively late in retinal development (Prada et al., 1991; Cepko et al., 1996). Work by Linser et al. (1997) suggests that mitotically active cells of the late embryonic retina express the Müller cell marker 2M6, and thus already possess glial-like qualities. Furthermore, they found a strong bias toward glial maturation that increased with age, similar to that seen in the cortex (Malatesta et al., 2000), suggesting that Müller cells may divide to produce the neuronal cell types of the retina, thus acting as neuroepithelial cells, prior to assuming their glial functions in adult life. They also appear to be the only cells in the chick retina capable of proliferation throughout adult life (for review, see Reh and Levine, 1998). Recent experiments by Fischer and Reh (2001) show that Müller cells can de-differentiate, acquire a PC-like phenotype, and divide to produce new neurons and glia following neurotoxic injury to the chicken retina. Two days after treatment with NMDA, Müller cells re-enter the cell cycle. The majority migrate to the ONL where they undergo a single round of division. During this process they lose their Müller cell phenotype and express proliferating cell nuclear antigen (PCNA) and transcription factors common to retinal PCs, including Pax6, Chx10 and CASH-1. The progeny of these Müller cell divisions includes cells that stain positive for neuronal or glial markers, although Fischer and Reh failed to find expression of markers for neuronal cell types other than bipolar and amacrine cells. This latter observation is consistent with the idea that the postnatal chick retina lacks the appropriate signals to generate all cell types. It is also unclear whether these new neurons become incorporated into the synaptic circuitry of the retina, and whether the de-differentiated Müller cells are able to return to their former glial state. However, along with the observations above, these experiments lend support to the idea that Müller cells possess a proliferative capacity that permits the production of multiple cell types within the developing and adult retina.
1.5 Extrinsic and intrinsic mechanisms regulate cell proliferation and differentiation in the retina

Neuronal cells and their PCs depend on signals from the intracellular and extracellular environments to guide their development. Several secreted factors have been shown to influence PCs towards different fates. Sonic hedgehog (Shh) appears to be responsible for initiating the differentiation of RGCs, which in turn start to secrete Shh (Zhang and Yang, 2001). Epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and leukaemia inhibitory factor (LIF) have also been shown to stimulate the production of certain retinal cell types whilst inhibiting the production of others (for review, see Lillien, 1998). The actions of these extracellular factors are not just restricted to cells in the proliferative cycle. Ciliary neurotrophic factor (CNTF) can re-direct immature rod photoreceptors towards a Müller cell fate after the final cell division (Ezzeddine et al., 1997). Contact mediated interactions, such as those involving the transmembrane proteins Notch and Delta, also play a critical role in the generation of neuronal diversity in the vertebrate retina (for review, see Perron & Harris, 2000). High levels of Notch signalling within a cell are thought to promote the retention of PC identity.

The regulation of cell proliferation and fate determination is not simply due to retinal PCs responding to an ever-changing environment of extracellular cues. A variety of cell types are born in overlapping phases, and if the above were the case, embryonic PCs cultured with postnatal retinal cells would be expected to adopt the fate of late born cell types, as the environment would contain the cues for late-born phenotypes. However, this does not occur. Similarly, culturing postnatally dividing PCs with an excess of embryonic cells would be expected to induce these PCs to differentiate into cell types more typical of early development such as RGCs. Whilst there is an inhibition of the production of the late-born cell types in these conditions, there is no promotion of the earlier born types (for review, see Cepko, 1999). Furthermore, there is a change in the ability of retinal PCs to respond to the various factors described above, as they progress through neurogenesis (for review, see Lillien, 1998). In addition to extrinsic signals, cell autonomous mechanisms also operate to mediate changes in the intrinsic responsiveness of a given PC to particular environmental signals. A current hypothesis proposes that during progression through neurogenesis, cell-intrinsic signals cause retinal PCs to progress through different competence states. The details of how this is achieved in
molecular terms are the area of intense current research and are beyond the scope of this introduction (reviewed by Marquardt and Gruss, 2002; Dyer and Cepko, 2001). One possible mechanism for mediating these changes involves changes in the expression of cell surface receptors that would allow PCs exposed to the same extracellular signals to respond differently.

There is growing evidence for the involvement of neurotransmitters in the regulation of cell proliferation. The vast majority of neurotransmitter receptors found in the brain are also found in the retina. Many in vitro studies have implicated a role for neurotransmitters, prior to the formation of synapses. Acetylcholine (ACh), acting via muscarinic receptors (mAChRs), can stimulate cortical precursor cell proliferation (Ma et al., 2000; Li et al., 2001), and purine nucleotides and nucleosides can increase or decrease DNA synthesis in glia and neurons (Ciccarelli et al., 1994, Sugioka et al., 1999b). GABA and glutamate have also been reported to be able to both increase (Fiszman et al., 1999; Haydar et al., 2000) and decrease (LoTurco et al., 1995) cell proliferation and GABA partially blocks the mitogenic actions of bFGF in the cortex (Antonopoulos et al., 1997). Both glutamate and GABA increase the size of cortical VZ clones, while decreasing sub-ventricular zone (SVZ) clone size (Haydar et al., 2000). In the cortex, LoTurco et al. (1995) have shown that, when applied alone, GABA\textsubscript{A} and AMPA/kainate receptor antagonists increase proliferation by increasing DNA synthesis, while when applied together they decrease it. The following section (1.6) briefly reviews cholinergic, purinergic, GABAergic and glutamatergic neurotransmitter systems. Section 1.7 describes current knowledge concerning some of the functions of neurotransmitter systems during development.

1.6 Classification and localization of neurotransmitter receptors

1.6.1 Cholinergic neurotransmission

ACh is the classic fast excitatory neurotransmitter of the peripheral nervous system. Its receptors can be divided into one of two categories: i) nicotinic-AChRs (nAChR), which are ionotropic receptors that are selectively activated by nicotine-like ligands and ii) mAChRs, which are metabotropic receptors, selectively activated by muscarine-like compounds. The action of ACh at the synapse is terminated predominantly by cleavage
through the action of the enzyme acetylcholinesterase (AChE). In addition, the early embryonic avian retina contains a significant level of butyrylcholinesterase (BuChE; Layer et al., 1987; for review, see Layer 1991), which also cleaves ACh (see Rang et al., 1999). These enzymes may be inhibited by the drugs eserine and iso-OMPA (tetraisopropyl pyrophosphoramide), respectively.

\[ \text{a) Nicotinic receptors} \]

The nAChRs belong to the ligand-gated ion channel family and are permeable to Na\(^+\) and K\(^+\). Depending on location they are termed muscle nAChRs and neuronal nAChRs. Each group is further characterised by their subunit composition and pharmacology. Neuronal nAChRs are generally heteromeric compositions of \(\alpha\) and \(\beta\) subunits. Molecular cloning has identified at least nine \(\alpha\) (1-9) and four \(\beta\) (1-4) subunits. Only the \(\alpha7\), \(\alpha8\) and \(\alpha9\) subunits form homomeric channels (for review, see Lindstrom, 1996) and they exhibit a high conductance for Ca\(^{2+}\) (Seguela et al., 1993). Antagonists include \(\beta\)-tubocurarine, hexamethonium and the classic neuromuscular blocker, \(\alpha\)-bungarotoxin (although this is now known to be ineffective in the case of the \(\alpha7\)-\(\alpha9\) homomers).

\[ \text{b) Muscarinic receptors} \]

The five sub-types of mAChR belong to the superfamily of membrane-bound receptors that contain seven transmembrane domains and elicit cellular responses via coupling with G-proteins (review, Nathanson, 1987). Five distinct isoforms have been characterised by molecular and pharmacological means. They all share common features including sensitivity to the agonists ACh and carbamylcholine (carbachol, CCh) and the classical antagonist atropine. Each receptor subtype couples to specific second messengers through an intervening G-protein. \(M_1\), \(M_3\) and \(M_5\) receptors stimulate phosphoinositide metabolism and the production of inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG) via phospholipase C (PLC), and cause Ca\(^{2+}\) release from IP\(_3\)-sensitive stores. The \(M_2\) and \(M_4\) receptor subtypes reduce cAMP levels via inhibition of adenylate cyclase (AC).
Muscarinic antagonists show some selectivity for the different receptor subtypes. For example, the M₁ receptor is selectively blocked by pirenzepine, the M₂ receptor by AFDX-116 (Ostenzepad / 11-[[2-[(Diethylamino) methyl]-1-piperidinyl] acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4] benzodiazepin-6-one) and the M₃ receptor by pF-HHSiD (p-fluoro-hexahydro-sila-difenidol). However, the mAChRs expressed in the chick are pharmacologically, immunologically and biochemically distinct from those expressed in mammalian tissues. Four avian mAChR subtypes have been characterised and named according to their sequence homology with the mammalian counterpart: cm2 (Tietje and Nathanson, 1991), cm3 (Gadput and Galper, 1994), cm4 (Tietje et al., 1990) and cm5 (Creason et al., 2000). The cloned cm2 receptor has been reported to have an anomalously high affinity for pirenzipine (Tietje et al., 1991). A threonine residue in the third outer loop of the cm2 receptor appears to be responsible for this anomaly. When the alanine at position 401 in the human M₂ receptor is mutated to threonine, it too presents increased pirenzipine sensitivity (Ellis and Seidenberg, 2000).

c) Localization of ACh and its receptors in the adult and developing retina

Cholinergic innervation of the eye is seen in all vertebrates. The choroid, iris and ciliary muscles all receive parasympathetic innervation. Immunocytochemical and biochemical studies have shown that ACh is a neurotransmitter in a subpopulation of amacrine cells, called ‘starburst’ cells (Baughman and Bader, 1977; Hayden et al., 1980), which are found in almost all vertebrate retinae, including birds (Hayes, 1984). The somata of starburst amacrines are found in both the INL and displaced within the GCL. Those located in the INL have dendrites in the OFF sublaminar of the IPL, whilst those located in the GCL have dendrites located in the ON sublaminar layer of the IPL.

nAChRs are expressed extensively in the INL and GCL of the rat and the goldfish retina (Wada et al., 1989; Cauley et al., 1990). In the chick retina, the α3, α7, α8 and β2 subunits are expressed on amacrines and RGCs (Keyser et al., 1988). mAChR binding sites have been identified in the retina of humans and monkeys (Zarbin et al., 1986) and ferrets (Hutchins, 1994). In the chick, cm2, cm3 and cm4 RNA and protein have all been found in retinal tissue extracts from both embryonic (see Figure 1.6) and hatched chick (McKinnon and Nathanson, 1995). Using antibodies directed to these same three chick mAChRs, Fischer et al. (1998a) labelled sections and extracts from ocular tissues,
including the retina and the RPE, of postnatal day 7-14 chicks. Within the retina, cm2 was expressed in numerous amacrines and RGCs; cm3 was found in many bipolar cells and a small subset of amacrine cells; and cm4 was present in the large majority of amacrines and RGCs. Cm3 immunoreactivity was also found in the RPE and choroidal extracts. The pattern of expression of the mAChR subtypes in the chick retina changes during development. Nadler et al. (1999) examined the developmental regulation of mAChR expression in the chick retina using immunoprecipitation and immunoblot analyses. Early in development, cm4 is the predominant subtype. Expression of the cm3 receptor appears to increase moderately, and that of cm2 dramatically, during the 2nd week of development (E7-14). The identity of the cell types in which these receptors are expressed has not been identified, and could include PCs as well as NDCs. Staining for markers of cholinergic neurons, such as AChE, may be observed as early as E3 in the chick retina (Layer et al., 1986; Layer, 1991). The level of expression of particular receptor sub-types can be altered by the addition of the culture medium from mature retinal cultures. A potentially novel and developmentally regulated secreted factor may cause a specific precocious expression of the cm2 receptor (Belmonte et al., 2000). This factor, called MARIA (Muscarinic Acetylcholine Receptor Inducing Activity), is produced by Müller cells. This is an example of the way in which differentiated cell types may signal information back to more immature cells, including PCs.

1.6.2 Purinergic neurotransmission

Purine bases and nucleotides are ubiquitous molecules found in all cells. As well as their well-known roles as information coding molecules in DNA and in cellular energy metabolism, these molecules are now known to play important roles as neurotransmitters and neuromodulators in the nervous system. Potential sources of extracellular purines include neurons, glia and microglia. ATP is co-released with other neurotransmitters from the synaptic vesicles of adrenergic, cholinergic, glutamatergic and GABAergic neurons (for review, see Burnstock, 1999). Purine nucleosides may also accumulate in the extracellular space as a result of bi-directional nucleoside transporters or ecto-nucleotidases (for review, see Rathbone et al., 1999). P1 receptors are activated by adenosine and its derivatives, whilst ATP and its derivatives stimulate P2 receptors. Once released the action of ATP may be stopped by i) its degradation by ecto-nucleotidases to adenosine diphosphate (ADP), adenosine monophosphate (AMP)
and adenosine (all themselves pharmacologically active), ii) by cellular re-uptake or iii) via receptor desensitisation and down-regulation.

a) **P2Y receptors**

P2Y receptors are G-protein coupled receptors that mediate a wide range of effects in response to adenine and uridine nucleotides (Filtz et al., 1997). They are coupled to PLC and IP$_3$ generation and the mobilisation of Ca$^{2+}$ from intracellular stores. There is increasing evidence to show that they also regulate cAMP and stimulate tyrosine kinases and mitogen activated protein kinases (MAPKs). Five subtypes are present in human tissue: P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$ and P2Y$_{11}$. The majority of these have been found in other mammals and the cloning of a chick brain cDNA library has led to the characterisation of an additional P2Y receptor, termed the p2y3 receptor (Webb et al., 1996), which is 65% homologous with the P2Y$_6$ receptor.

The pharmacology of purinergic receptors is complex, and there are currently few agonists or antagonists with a high selectivity, and none that are specific for a given subtype. Determination of receptor subtype requires an assessment of agonist potencies and a profile of antagonist inhibition. Even this is not without problems; individual recombinant receptor subtypes can display alternative agonist profiles when expressed in different host cells. For example, the agonist profile of the recombinant p2y3 receptor expressed in Xenopus oocytes was found to be ADP>UTP>ATP>UDP. In contrast, p2y3-expressing Jurkat cells have a potency order of UDP>UTP>ADP>ATP (see Li et al., 1998). The pharmacological profile also varies between species (e.g. Kennedy et al., 2000).

The P2Y$_1$ receptor is activated selectively by adenine nucleotides, with ADP being more potent than ATP (Schachter et al., 1996). The P2Y$_2$ receptor is stimulated equally by ATP and by uridine triphosphate (UTP) (Parr et al., 1994). Nguyen et al. (1995) suggested that the P2Y$_4$ receptor is activated by UTP alone, although a more recent study of the cloned rat P2Y$_4$ receptor suggest it too can be equally sensitive to both UTP and ATP (Bogdanov et al., 1998). The avian homologue of the P2Y$_4$ also exhibits activation by both UTP and ATP (Boyer et al., 1997). The P2Y$_6$ receptor is specifically
activated by uridine diphosphate (UDP) (Nicholas et al., 1996), and ATP activates the P2Y11 receptor (Communi et al., 1997a).

The majority of purinergic antagonists act on more than one receptor type. The only specific blocker known to date is MRS 2179, which is a potent antagonist of the P2Y1 receptor. Suramin is a broad-spectrum purinergic antagonist blocking the P2Y1, P2Y2, p2y3, P2Y6 and P2Y11 receptors. However, it is ineffective at the P2Y4 receptor (Charlton et al., 1996). Similarly, Reactive Blue blocks all bar the P2Y2 subtype although it is only a partial antagonist at the P2Y11 receptor. PPADS (pyridoxal phosphate-6-azophenly-2’, 4’-disulphonic acid) inhibits the P2Y1 and P2Y6 receptors. There are conflicting reports concerning the effects of PPADS at the P2Y4 receptor: Communi et al. (1996b) found that the cloned receptor response to UTP was strongly inhibited by PPADS yet Charlton et al. (1996) found the same antagonist to be ‘relatively ineffective’ despite the fact that both researchers used the same cell line (1321N1 astrocytoma cells). Its efficacy at the p2y3 receptor has yet to be established.

b) P2X receptors

P2X receptors belong to the ligand-gated family of ion channels and are multimers formed via polymerisation of P2X subunits. Opening of the ion channel by ATP or other agonists permits the passage of Na+, K+ and Ca2+ and causes membrane depolarisation and further Ca2+ influx. Based on agonist efficacy and desensitisation characteristics, P2X receptors have been grouped into three distinct classes (Dubyak et al., 1996). Group 1 (P2X1 and P2X3) have a high affinity for ATP, and are rapidly activated and desensitised; Group 2 (P2X2, P2X4, P2X5 and P2X6) have a ten fold lower affinity for ATP and show slower desensitisation; Group 3 consists of just the P2X7 receptor which has very low affinity for ATP, does not desensitise and can act as a non-selective ion pore. P2X receptors are relatively insensitive to activation by UTP. There are few selective P2X receptor antagonists although TNP-GTP (2’, 3’)-O- (2,4,6-trinitrophenyl) guanosine 5’-triphosphate) has been shown to be a potent antagonist at the P2X1 and P2X3 receptors. All seven P2X receptor subunits are found in the CNS.
ATP and adenosine play important roles in development from the moment of fertilisation. In the chick, along with mAChRs, ATP receptors are among the first functionally active membrane receptors, present at the time of germ layer formation (Laasberg, 1990). Purinergic agonists have been reported to influence cell proliferation, apoptosis and DNA synthesis throughout the early stages of development (Figure 1.6). There is little systematic data on the developmental expression patterns of the different receptor subtypes. There are few commercial sources of purinergic antibodies (currently antibodies for the rabbit P2Y1, P2Y2 and P2Y4 receptors are available) and the majority of studies have relied on hybridisation immunohistochemistry. The P2Y1 receptor is expressed in a developmentally regulated manner in the chick limb buds, brain, somites and facial primordia (Meyer et al., 1999). In the retina there is a dramatic decline in ATP-induced changes in \([\text{Ca}^{2+}]_i\) prior to synaptogenesis (Sakaki et al., 1996), a decrease that parallels the reduction in mitotic activity seen during retinal development. Rat retinal glial cells express P2Y2 and P2Y4 receptors. P2X receptors have been identified in rat RGCs (Wheeler-Schilling et al., 2001) and in the Müller cell population of the human retina (Pannicke et al., 2000). P2X2, P2X3, P2X4 and P2X7 have all been found in the mammalian retina (Greenwood et al., 1997; Wheeler-Schilling et al., 2001; Brändle et al., 1998).

1.6.3 GABAergic neurotransmission

GABA is the major inhibitory neurotransmitter of the mammalian CNS, and in the chick retina it is located predominantly in amacrine and horizontal cells. It is released via Ca\(^{2+}\)-dependent exocytosis, or in some cases via the action of GABA transporters (Schwartz et al., 1987). The action of GABA is terminated by its removal from the extracellular space via a class of Na\(^+\)-dependent GABA transporters (GAT1-4; Guastella et al., 1990, Liu et al., 1993). \textit{In situ} hybridisation techniques have revealed GAT1 is present in the rat and mouse retinas. It has been located in amacrines and some RGCs, and is expressed at low levels in Müller cells (Brecha and Weigman, 1994; Ruiz et al., 1994).
a) **GABA receptors**

There are three types of GABA receptor termed GABA\(_A\), GABA\(_B\) and GABA\(_C\) receptors. GABA\(_A\) and GABA\(_C\) receptors belong to the ligand-gated ionotropic receptor superfamily. There is great diversity amongst GABA\(_A\) and GABA\(_C\) receptors. They differ in terms of their kinetic properties, affinity for agonists and antagonists and sensitivity to modulators (for review, see Rudolph et al., 2001).

Ionotropic GABA receptors are pentameric complexes of subunits. There are several families of subunits, known as \(\alpha, \beta, \gamma, \delta, \varepsilon, \pi\) and \(\rho\). The GABA\(_A\) receptor complex includes \(\alpha\) subunits and GABA\(_C\) receptors include \(\rho\) subunits. Multiple variants exist within each class and different subunit combinations produce receptors with different properties. Activation of the GABA\(_A\) receptor opens an integral chloride (Cl\(^-\))-permeable channel, and causes the cell to hyperpolarize. During development, however, the action of GABA is to cause depolarisation (Segal and Barker 1984). In neonatal hippocampal neurones, synaptically released or exogenously applied GABA depolarises and excites neuronal membranes (for review, see Cherubini et al., 1991). This effect is believed to be due to the high [Cl\(^-\)]\(_i\) found in these cells relative to that in mature neurons, and is a common feature in developing systems. It is suggested that during development GABA exerts mainly a trophic action through membrane depolarisation and a rise in [Ca\(^{2+}\)]\(_i\). The GABA\(_A\) receptor is selectively stimulated by muscimol and inhibited by bicuculline. Like the GABA\(_A\) receptor, the GABA\(_C\) receptor is linked to a chloride channel. However, it is insensitive to bicuculline but may be activated by the GABA analogue cis-4-aminocrotonic acid. Picrotoxin is a non-competitive antagonist of virtually all classes of ionotropic GABA receptor (for review, see Whiting et al., 1995). GABA\(_B\) receptors are linked indirectly to Ca\(^{2+}\) and K\(^+\) channels via G-proteins, are selectively activated by baclofen, and are insensitive to bicuculline.

b) **Localization of GABA and its receptors in the adult and developing retina**

GABA is released in the adult retina by amacrine and horizontal cells, which mediate lateral inhibition within the retina, and all three GABA receptor subtypes are found in the retina. GABA is synthesised mainly from glutamate by the glutamic acid decarboxylase enzyme (GAD). Da Costa Calaza et al. (2000) examined the
developmental changes in GAD expressing neurons. Whilst there is some expression (20%) between E3 and E6, the levels of GAD (as revealed by immunohistochemical staining) rapidly increase from E6, a time that corresponds with the production of amacrine and horizontal cells.

Immunohistochemical labelling has demonstrated that GABA<sub>A</sub> receptors are present in the IPL and OPL of the ferret retina (Karne et al., 1997). In the rabbit, mRNA for the GABA<sub>A</sub> α1, β2, β3 and γ2 subunits is expressed in sub-populations of bipolar, amacrine and RGCs (Greferath et al., 1994). GABA<sub>A</sub> receptors are also expressed very early on in development (see Figure 1.6), prior to synaptogenesis. Ca<sup>2+</sup> imaging studies have shown that the chick retina undergoes changes in [Ca<sup>2+</sup>]; in response to GABA - a response that may be blocked by GABA<sub>A</sub> antagonists (Yamashita and Fukuda, 1993). GABA<sub>B</sub> receptors and the GABA transporter GAT-1 are present from the onset of synaptogenesis, at E8, and are located on RGCs and amacrine cells (Catsicas and Mobbs, 2001). GABA<sub>C</sub> receptor subunits were first detected in the mouse retina at postnatal day (P) 6 and functional receptors are present by P9 (Greka et al., 2000). This correlates with the period of bipolar cell differentiation as well as eye opening.

Autoradiographic studies of GABA uptake in the E6 chick retina show labelling of cell bodies and processes in the central regions of the retina, but no labelling in the VZ (Frederick 1987). At E8, horizontal cells, amacrine cells and RGCs all labelled positive for GABA uptake, although dividing cells remained unlabelled. In the ferret, GABA immunoreactivity is seen in the GCL, amacrine cells and the IPL by E38 although in contrast to most other species, the OPL stains only weakly (Karne et al., 1997).

1.6.4 Glutamatergic neurotransmission

Glutamate is the predominant fast excitatory neurotransmitter of the CNS. Glutamate is released via Ca<sup>2+</sup>-dependent exocytosis and its action is terminated by removal from the extracellular space via Na<sup>+</sup>-dependent transporters. Glutamate is preferentially taken up by Müller cells in the retina (White and Neal, 1976). The actions of glutamate are mediated by ionotropic and metabotropic receptors. For a more comprehensive review of glutamate receptors see Brandstatter et al., (1998). Ionotropic glutamate receptors
contain glutamate-gated cation-specific ion channels, whereas metabotropic receptors are coupled to G proteins.

a) Ionotropic glutamate receptors

There are three classes of ionotropic glutamate receptors composed of different subunits: AMPA (α-amino-3-hydroxy-5-methyl-4-isooxasolepropionate; GluR1-4), KA (kainate; GluR5-7 and KA1-2), and NMDA (N-methyl-D-aspartate; NR1, NR2A-D). Each receptor is comprised of four subunits, and any combination of subunits from a given class can form heteromeric complexes in vivo.

The NMDA receptor is linked to a cation channel that is highly permeable to Na\(^+\), K\(^+\) and Ca\(^{2+}\). The Ca\(^{2+}\) permeability of this receptor underlies its roles in development, cell cytotoxity and in learning and memory. The channel is blocked by magnesium (Mg\(^{2+}\)) at negative membrane potentials. This block is voltage-dependent, preventing the passage of ions at voltages near the resting potential, and is relieved by depolarisation. Glycine is required for channel opening, binding to an allosteric site and so acting as a co-agonist. NMDA receptors have a high affinity for glutamate, activate relatively slowly and display slow deactivation and desensitization rates. NMDA, glutamate, ibotenate and L-aspartate are all efficacious agonists of the NMDA receptor. Antagonists include, amongst others, MK-801, D(-)-2-amino-5-phosphonopentoic acid (D-AP5) and zinc.

AMPA/kainate receptors mediate fast excitatory neurotransmission throughout the CNS. They also permit the entry of Na\(^+\) and K\(^+\) through cation channels but unlike the NMDA receptor have low permeability to Ca\(^{2+}\) (but see later). AMPA receptors have a high affinity for glutamate, activate rapidly and deactivate and desensitise quickly in the presence of the agonist. However, application of kainate evokes a long-lasting current that persists in the continued presence of the drug (Patneau et al., 1992). Glutamate-evoked Ca\(^{2+}\) entry may result from membrane depolarisation leading to Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels (VGCCs). However, in certain circumstances, AMPA/kainate receptors are highly permeable to Ca\(^{2+}\). Four AMPA/kainate receptor subunits have been identified (GLUR1-4) and these may form homomeric or heteromeric channels. Each of these subunits may be expressed as one of two forms termed ‘flip’ and ‘flop’. These differ in a region of 38 amino acids located in the section
that precedes the last transmembrane spanning part of the subunit. Channels expressing the flip variant have a higher affinity for glutamate than the flop form. The expression of flip and flop changes during development with flip predominating at early times, the flop form not appearing until later in development. The higher affinity of the flip variant for glutamate may be important in the establishment of synapses during development. The subunit GLUR2 is particularly important in determining channel characteristics; channels that include the GLUR2 subunit have a low Ca\(^{2+}\) permeability whilst those in which the GLUR2 subunit is absent show a high Ca\(^{2+}\) permeability. Quisqualate and kainate are effective agonists at the AMPA receptor along with glutamate and AMPA. The AMPA/kainate receptor is inhibited by quinoxalinediones including CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), DNQX (6,7-dinitro-quinoxaline-2,3-dione) and the more efficacious NBQX (1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide).

b) **Metabotropic glutamate receptors**

Metabotropic glutamate receptors (mGluRs) are coupled to G-proteins and are subdivided into three groups according to their sequence homology, pharmacology and signal transduction mechanisms. Molecular cloning techniques have currently identified eight subtypes. mGluR1 and 5 belong to group 1 and are coupled to IP\(_3\)/Ca\(^{2+}\) signal transduction and the production of arachadonic acid and cAMP (Aramori and Nakanshi, 1992). Group 2 includes mGluR 2 and 3, which reduces the production of cAMP by inhibiting adenylate cyclase. Group 3 is comprised of mGluR4, 6, 7 and 8, which also inhibit adenylate cyclase. Agonists at the mGluR include L-glutamate, ibotenate and quisqualate. Specific agonists include 3,5-dihydroxiphenylglycine (DHPG), which acts at Group 1 receptors, (2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate (2R,4R-APDC) acting on Group 2 receptors and L-2-amino-4-phosphonobutyric acid (L-AP4), an analogue of glutamate, which strongly activates Group3. L-2-amino-3-phosphonopropionic acid (L-AP3) is an antagonist at most metabotropic glutamate receptors.
c) Localization of glutamate and its receptors in the adult and developing retina

Glutamate is the main neurotransmitter of RGCs, bipolar cells and photoreceptors in the vertebrate retina. The distribution of ionotropic glutamate receptors has been described in the retina of several species although there is relatively little data for the chick. The density of AMPA/kainate receptors in the rat forebrain is greatest during development and declines at later times (Miller et al., 1990). Electrophysiological recordings from retinal cell cultures, and \( \text{Ca}^{2+} \) imaging studies in whole mount retinæ, have shown that \( \text{Ca}^{2+} \) permeable forms of the AMPA/kainate receptor are present in the chick retina by E6 (Allcorn et al., 1996). Pellegrini-Giampietro et al., (1992) demonstrated that the ratio of the mRNAs of the \( \text{Ca}^{2+} \) permeable to \( \text{Ca}^{2+} \) impermeable subunits decreases with age in the rat brain. Together, these studies indicate a specific developmental role for the \( \text{Ca}^{2+} \) permeable form of the AMPA/kainate receptor. Interestingly, Marie et al. (2000) present data from embryonic rat cortex that suggests functional ionotropic glutamate receptors are expressed at the time of terminal cell division and early differentiation. Whole-cell patch clamping and \( \text{Ca}^{2+} \) imaging revealed that glutamate-evoked inward currents and associated increases in \([\text{Ca}^{2+}]_i\) are only seen in differentiating neurons. In contrast, PCs failed to respond to any ionotropic agonist. However, a subpopulation of cells that stained positively for both BrdU (a marker of proliferation) and TuJ-1 (an antibody against neuron-specific \( \beta \) tubulin and a marker of differentiated neurons) did show increases in \([\text{Ca}^{2+}]_i\) in response to AMPA, and Marie et al. propose that glutamate receptors emerge during terminal cell division in the rat cortex.

GLUR1-7 are present in the adult retina of both rat and cat (Hamassaki-Britto et al., 1993). In the postnatal chick, all the subunits of the AMPA/kainate receptors and the NR1 subunit of the NMDA receptor are found in cells of the INL and the GCL, mostly on amacrines and RGCs (Santos Bredariol and Hamassaki-Britto, 2001). All the aforementioned subunits were also found at the earlier age of E5, and although staining was predominantly restricted to the GCL, some positive staining was observed in other regions of the retina. Distribution of the mGluR within the CNS has not been as widely investigated although mRNA for mGluR4, 6 and 7 has been detected in the rat retina (Akazawa et al., 1994). In the adult retina, the mGluR6 receptor is found almost exclusively on the ON-type bipolar cells (Shiells and Falk, 1990). The mGluR6 is linked to a G protein-coupled phosphodiesterase. A reduction in the release of glutamate
from rods following illumination inactivates the phosphodiesterase (see section 1.2),
causing [cGMP] \(_j\) to rise and the ON bipolar cell to depolarise, augmenting the release of
glutamate from the ON bipolar cell and exciting the ON pathway. The OFF bipolar cell
expresses AMPA/kainate receptors at the synapses with rods. A reduction of glutamate
release from rods onto OFF bipolar cells causes a hyperpolarization and a lower rate of
release of glutamate on to the OFF RGCs (Nawy and Jahr, 1990; Shiells and Falk,
1990).

1.7 Roles of neurotransmitters in development

During development neurotransmitters act as growth regulatory signals to control cell
proliferation, differentiation, and gene expression by activating both voltage-gated
channels and receptors coupled to specific second messenger pathways (Lauder, 1993).
Even prior to their final division, many cortical and retinal PCs express functional
receptors for the principal excitatory and inhibitory transmitters and these systems can
exert powerful effects in the regulation of cell proliferation.

Muscarinic agonists cause increases in \([Ca^{2+}]_i\) in cells of the VZ in the rabbit retina. In
contrast, nicotinic agonists are more effective at inducing rises in \([Ca^{2+}]_i\) in RGCs and
amacrine cells (Wong, 1995). The muscarinic response disappears at the end of cell
division and thus raises the possibility that muscarinic stimulation acts to promote
continued cell division, a view supported by the discovery of the mitogenic effects of
mAChRs on astrocytes in the CNS. Muscarinic agonists cause increases in \([Ca^{2+}]_i\) in
astrocytes and the induction of the immediate early genes c-fos and c-jun (Trejo and
Brown, 1991). Ashkenazi et al. (1989) found that activation of the M\(_1\), M\(_3\) and M\(_5\)
subtypes induce proliferation in rat cortical astrocytes. ACh, acting via mAChRs, can
also stimulate cortical precursor cell proliferation (Ma et al., 2000; Li et al., 2001). The
downstream actions of receptor activation are mediated by the activation of MAPKs
(Ma et al., 2000), extracellular regulated kinases (Erks) and phosphatidylinositol-3
Kinase (PI-3K; Li et al., 2001). In other cell systems, mAChR stimulation can lead to an
inhibition of proliferation. When M\(_1\) and M\(_3\) mAChRs are expressed in A9 L cell lines,
the muscarinic agonist CCh causes a reduction in DNA synthesis as measured by 3H-
thymidine incorporation (Conklin et al., 1988). Similarly, activation of M\(_3\) mAChRs in
small cell lung carcinoma cells causes their arrest in S and G2/M phases of the cell cycle (Williams and Lennon, 1991).

Purine nucleotides and nucleosides can increase or decrease DNA synthesis in glia and neurons (Ciccarelli et al., 1994, Sugioka et al., 1999b). ATP increases 3H-thymidine incorporation in retinal cultures from E3 while the antagonists suramin and PPADS inhibit these effects (Sugioka et al., 1999b). ATP and UTP also produce changes in [Ca2+]i in whole mount chick retinae of the same age (Sugioka et al., 1996). However, ATP can produce different effects in the proliferation rate of cultured rat astrocytes, depending on the exposure time. Ciccarelli et al. (1994) found that cell proliferation was increased when exposure to ATP was limited to 8h, but was decreased when the exposure time was extended to 16h.

The presence of GABA and its receptors early in the development of the vertebrate nervous system has led to several recent investigations into their role in PC proliferation, migration and differentiation. GABA promoted differentiation in cultures of cortical and retinal neurons from the chick (Spoerri, 1988) and has also been shown to promote proliferation in cultures of immature cerebellar granule cells (Fiszman et al., 1999). However, GABA can partially block the bFGF-induced increase in cortical proliferation in vitro (Antonopoulos et al., 1999). LoTurco et al. (1995) demonstrated that cells from the rat neocortical VZ were depolarised by GABA and glutamate, and that this was accompanied by a concomitant increase in [Ca2+]i. Application of either GABA or glutamate resulted in a decrease in DNA synthesis and the authors suggest that these neurotransmitters cause an arrest in the cell cycle at the G1 to S-phase. Interestingly, when applied alone, GABA and AMPA/kainate receptor antagonists increased proliferation by increasing DNA synthesis, while when applied together they decreased it. Haydar et al. (2000) addressed the issue of whether GABA and glutamate act on all PCs in a similar manner or if their actions differ between brain regions. They found that both glutamate and GABA increase the rate of BrdU uptake and cortical VZ clone size, while causing a decrease in these measures in the adjacent sub-ventricular zone (SVZ). The cultures used in LoTurco’s experiments did not distinguish between these two regions. Indeed, Haydar et al. also found that GABA and glutamate could cause an apparent overall decrease in DNA synthesis when cells from the VZ and SVZ were pooled, but different effects when the two regions were analysed separately. Thus,
GABA and glutamate can stimulate or inhibit proliferation of embryonic PCs, the precise effects being dependent upon the source of the cells investigated.

Neurotransmitters have also been shown to be important in the migration of immature neurons (discussed in section 1.9), circuit formation (discussed in section 1.8.3) and neuronal outgrowth. Komuro and Rakic (1993) demonstrated the involvement of glutamate in the migration of cerebellar granule cells in the developing mouse cortex. Activation of NMDA receptors increased the rate of granule cell movement whilst antagonists caused a reduction. Mammalian retinal cells spontaneously release ACh, and inhibition of nAChRs leads to an increase in retinal neurite outgrowth in culture (Lipton et al., 1988). In rat cerebellar granule cells glutamate, acting via activation of NMDA receptors, stimulates neurite outgrowth (Rashid and Cambray-Deakin, 1992). Conversely, glutamate inhibits neurite outgrowth in embryonic chick retinal neurons and acts via Ca^{2+}-permeable AMPA receptors (Catsicas et al., 2001). Thus, the same neurotransmitter can have different effects that depend not only on the system studied but also on the receptors expressed by a given cell type.

1.8 Spontaneous [Ca^{2+}]_i transients and the regulation of neuronal development

Neuronal development is regulated by a vast array of mechanisms that include electrical activity and the production of second messengers such as cAMP and IP_3. Many of the signalling processes involved in these regulatory mechanisms result in a change in [Ca^{2+}]_i. The following section considers the role of [Ca^{2+}]_i in the regulation of the development of the CNS.

1.8.1 [Ca^{2+}]_i stores and release mechanisms

[Ca^{2+}]_i is involved in the control of many different cellular functions in development ranging from proliferation and differentiation to migration and neuronal outgrowth. [Ca^{2+}]_i may be increased by opening channels either in the plasma membrane or in the membrane of internal stores. Ca^{2+} can enter from the extracellular space via VGCCs, receptor-operated channels and Ca^{2+} release activated channels (CRACs). [Ca^{2+}]_i signalling can take the form of single transients, repetitive oscillations or sustained plateaus. These [Ca^{2+}]_i signals permit downstream effectors to decode information
contained in the amplitude, frequency and duration of these changes. IP$_3$ releases Ca$^{2+}$ by binding to IP$_3$ receptors (of which there are three isoforms) in the membrane of the endoplasmic reticulum. This release mechanism has two interesting properties relevant to the multiple functions of Ca$^{2+}$ in development: Ca$^{2+}$ release can be ‘quantal’ in nature and may show a bi-phasic sensitivity to cytosolic [Ca$^{2+}$] (Short et al., 2000). The former property arises from the discrete nature of Ca$^{2+}$ stores as pools with different IP$_3$ sensitivities. Given the strong spatial buffering of [Ca$^{2+}$]$_i$ within cells, this means that [Ca$^{2+}$]$_i$ can be raised at specific and discrete locations within cells. The bi-phasic sensitivity of the stores is due to their auto-inactivation via the binding of Ca$^{2+}$ to the IP$_3$ receptor. The effects of Ca$^{2+}$ are mediated by Ca$^{2+}$-binding proteins and by downstream effectors that include diverse targets such as phosphatases and kinases, and transcription factors. A detailed discussion of the mechanisms of [Ca$^{2+}$]$_i$ signalling can be found in the recent review by Bootman et al. (2001).

1.8.2 [Ca$^{2+}$]$_i$ and regulation of the cell cycle

The regulation of progression through the cell cycle is complex and involves, amongst other things, the activation of kinases and proteases, and production of 2nd messengers. Increasingly, Ca$^{2+}$ is becoming recognised as a key regulator of several checkpoints within the cell cycle (for review, see Santella, 1998). [Ca$^{2+}$]$_i$ transients have been observed in late G$_1$ prior to the initiation of S phase, in G$_2$ before entry into M phase, during mitosis at the transition from metaphase to anaphase and during cytokinesis. These transients lead to the activation of Ca$^{2+}$ binding proteins. One of the key targets is calmodulin, which stimulates CaM-kinase II and the CaM-dependent protein phosphatase calcineurin, both of which have been shown to intervene at the checkpoints described above. The protease calpain is activated by Ca$^{2+}$ and is responsible for the degradation of cyclin D1, which promotes both mitosis and meiosis when injected into somatic cells or oocytes (for review, see Santella et al., 1998). In chick retina, mobilization of Ca$^{2+}$ from intracellular stores and capacitative Ca$^{2+}$ entry has been shown to be essential in permitting DNA synthesis in retinal neuroepithelial cells (Sugioka et al., 1999a).
1.8.3 Spontaneous $[\text{Ca}^{2+}]_i$ activity in the developing nervous system.

a) Spontaneous changes in $[\text{Ca}^{2+}]_i$ in VZ cells

Despite an increasing awareness of the importance of changes in $[\text{Ca}^{2+}]_i$ in the regulation of the cell cycle and proliferation, the $\text{Ca}^{2+}$ dynamics of PCs have received relatively little attention. Owens and Kriegstein (1998) used confocal microscopy to examine the spatiotemporal patterns of spontaneous $[\text{Ca}^{2+}]_i$ changes in the embryonic rat cortical VZ. They observed a large number of cells undergoing single, low frequency transients that lasted for many seconds. These changes occurred randomly and were independent of action potentials and amino acid neurotransmitter receptor activation. Occasionally, paired events were observed, occurring in dividing cells during cytokinesis. The third pattern of $[\text{Ca}^{2+}]_i$ fluctuations consisted of a co-ordinated rise in groups of up to 20 neighbouring cells. Whilst they did not attempt to block these transients, Owens and Kriegstein propose that such cluster events arise from gap junctional coupling between groups of PCs and that this coupling may function to synchronize cell cycle events. Spontaneous increases in $[\text{Ca}^{2+}]_i$ have also been reported in gap junction-coupled neurons in the developing postnatal cortex (Yuste et al., 1992, 1995). More recently, the use of two-photon microscopy has demonstrated that previously unseen large-scale, synaptically mediated $[\text{Ca}^{2+}]_i$ oscillations propagate along the entire longitudinal axis of the developing cortex (Garaschuk et al., 2000). Similar large-scale activity involving neurotransmitters and receptors is seen in the visual system both before and after synapse formation (see section c, below).

b) Spontaneous $[\text{Ca}^{2+}]_i$ transients control neuronal differentiation and growth

$[\text{Ca}^{2+}]_i$ plays a major role in the regulation of neuronal differentiation and growth. Imaging studies have shown that multiple spontaneous $[\text{Ca}^{2+}]_i$ transients occur in developing Xenopus spinal neurons in vitro, and that they take one of two forms. The first, called a $\text{Ca}^{2+}$ spike, requires $\text{Ca}^{2+}$ influx through voltage-gated channels and $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$-release (CICR) (Gu and Spitzer, 1993; Gu et al., 1994). These relatively brief (~10s duration) and large signals are regenerative events, and thus propagate throughout the neuron. In contrast the second type of event, $\text{Ca}^{2+}$ waves, have slower kinetics (~30s) and do not involve $\text{Ca}^{2+}$ action
potentials. They occur locally and decay passively with distance (Gu and Spitzer, 1995). Waves require Ca$^{2+}$ entry through an unidentified channel and are amplified by Ca$^{2+}$ release from intracellular stores (Holliday et al., 1991). Spikes and waves control a variety of developmental events via information encoded in their frequency, amplitude and duration. Ca$^{2+}$ spikes have been found to regulate the development of K$^+$ currents, neurotransmitter receptor expression and the synthesis of GABA (Spitzer et al., 1993). Inhibition of spikes by an over-expression of K$^+$ channels leads to a reduction in the number of differentiated neurons in culture (Jones et al., 1994). It is possible that the transients observed in the cortex (Owens and Kriegstein, 1998, see above) also play a regulatory role in neurogenesis. In contrast to spikes, the spatially restricted nature of Ca$^{2+}$ waves enables control of local rather than global events. For example, the rate of neurite outgrowth is inversely related to the frequency of Ca$^{2+}$ waves in growth cones. Gu and Spitzer (1995) were able to replicate the effects of spontaneous Ca$^{2+}$ waves on the inhibition of neurite outgrowth by using high K$^+$ to induce [Ca$^{2+}$]$_i$ changes of the same frequency, kinetics and magnitude as occurs naturally. Replicating the normal frequency of the faster Ca$^{2+}$ spike activity, however, was ineffective showing that in growth cones the slower Ca$^{2+}$ waves are necessary and sufficient for regulating neurite outgrowth. These findings have been replicated in vivo (Gomez and Spitzer, 1999).

Suppressing [Ca$^{2+}$]$_i$ transients via photorelease of a Ca$^{2+}$ chelator causes an acceleration of axon extension in embryonic rat spinal chord neurons. Conversely, mimicking the transients through the photorelease of caged Ca$^{2+}$ slows the otherwise more rapid outgrowth of axons. Growth-cone stalling and axon retraction were also found to be associated with high frequencies of [Ca$^{2+}$]$_i$ transients. Subtle changes in [Ca$^{2+}$]$_i$ have been implicated in events such as growth cone turning. Zheng (2000) induced focal elevations in [Ca$^{2+}$]$_i$ at one side of the growth cone by repetitive uncaging of Ca$^{2+}$. These elevations lead to turning of the growth cone towards the side on which Ca$^{2+}$ is elevated.

How the kinetics and magnitude of [Ca$^{2+}$]$_i$ transients control different events such as movement and gene expression are not well understood. A specific explanation is suggested by the set-point hypothesis. This proposes that axon outgrowth is maximal within a narrow range of [Ca$^{2+}$]$_i$ and that levels above or below this optimum level cause a slowing or cessation of growth (Zimprich et al., 1994). Global and local changes in [Ca$^{2+}$]$_i$ produce different effects on neurite outgrowth (Gu and Spitzer, 1995). The
effects of \([Ca^{2+}]\) on gene transcription also depends on the frequency and magnitude of these changes (Dolmetsch et al., 1998) although the details of the molecular mechanisms underlying these processes remain to be determined.

Spontaneous changes in \([Ca^{2+}]\) occur in postmitotic neurons in the early stages of differentiation and migration. Changes in \([Ca^{2+}]\) occur in migrating cerebellar granule cells (Komuro and Rakic, 1996) and the rate of migration is positively correlated with the frequency and amplitude of these transients. These and other observations on the role of \(Ca^{2+}\) in cell migration are discussed in detail in section 1.9.

c) **Spontaneous \([Ca^{2+}]\) transients modulate visual circuit formation**

Co-ordinated spontaneous activity is a common feature of many parts of the developing nervous system (reviewed by Wong, 1999), and is particularly important in the developing visual system at the time of synapse formation. This activity takes one of two forms: spontaneous activity independent of sensory inputs and experience-driven activity. Developing circuits in the retina generate spontaneous and synchronized patterns of electrical activity before the animal has any visual experience. This is thought to contribute to the development of the retinal circuitry and the refinement of the synaptic connections within the lateral geniculate nucleus (LGN) and the primary visual cortex (Mooney et al., 1996; see also Wong, 1999).

Neurons in the inner retina show waves of spontaneous electrical activity that spread between neighbours, raising \([Ca^{2+}]\) as they pass. During the first two postnatal weeks in the ferret retina, these waves result from electrical activity synchronized between neighbours (e.g. Wong et al., 1995). Similar patterns of activity are seen in all RGCs and are propagated in the form of waves across the whole surface of the retina. These waves have been described for mammalian (Meister et al., 1991; Wong et al., turtle (Sernagor and Mehta, 2001) 1995) and chick (Catsicas et al., 1998; Wong et al., 1998) retina. RGCs receive synaptic input from cholinergic starburst amacrine cells, which also contain the neurotransmitter GABA, at the time this bursting activity occurs. From early times bursting activity involves cholinergic transmission (Feller et al., 1996; Penn et al., 1998; Sernagor and Grzywacz, 1996, 1999). However, at times depending on the species GABAergic, glycinergic and glutamatergic transmission also play a role in the modulation and
generation of these waves (Fischer et al., 1998; Wong et al., 1998; Catsicas and Mobbs, 2001; for review, see Wong, 1999). In the rabbit retina the cholinergic activity is initially mediated by nAChRs but in later stages the muscarinic system becomes the driving force (Zhou and Zhao, 2000). In the following two weeks, synapses form between the photoreceptors, bipolar cells and RGCs of the retinal vertical pathway. At the same time, RGCs begin to differentiate into ON and OFF subclasses; their dendritic connections stratify into different regions within the IPL, and their axon terminals become restricted to different sublaminae in the LGN. During this time, ON and OFF RGCs begin to develop different bursting patterns and undergo a developmental shift from cholinergic to glutamatergic initiation (Wong et al., 2000), a change that has also been observed in the chick retina (Catsicas et al., 1998; Wong et al., 1998). Furthermore, during the transition between the two systems, each modulates different properties of the waves and hence may play specific instructive roles in visual development. Semagor et al. (2000) demonstrated that in the chick, glutamate acts on wave velocity whilst ACh influenced the recruitment of cells and hence the spatial aspect of waves. Thus, the nature of correlated spontaneous activity, induced by neurotransmitter inputs, undergoes developmental shifts as new cellular components are added to the retinal circuitry and these distinct patterns of activity play important roles in the segregation of RGC terminals in the LGN.

Early in development the terminals of RGC axons from left and right eye overlap in the LGN. Later on, this overlap is eliminated and the terminals of left and right eye RGCs form distinct and separate layers. The current model for this sorting process is one in which axons compete with one another to establish synaptic contacts with target neurons (Shatz, 1990). This requires RGC activity since blocking RGC action potentials with TTX prevents correct layer formation in the LGN (see review by Shatz, 1996). The initial projections of RGCs to the LGN are refined as simultaneously active inputs strengthen their connections on to common postsynaptic neurons at the expense of weakly or asynchronously active ones. As a result, synchronously active RGCs will innervate common targets whilst those that fire asynchronously will be weakened and eliminated (for review, see Miller, 1996).

Co-ordinated spontaneous activity in the retina does not always depend on synaptic transmission since [Ca\(^{2+}\)] transient are seen at times prior to synapse formation. In the
early (E8-E11) chick retina, Ca\(^{2+}\) wave activity appears to propagate via gap junctions and can be blocked by the gap junction blockers octanol and 18\(\alpha\) glycyrrhetinic acid (Catsicas et al., 1998). Endogenous ACh, dopamine, glycine and other neurotransmitters modulate the temporal pattern of the activity suggesting that the mechanisms responsible for the triggering and propagation of \([Ca^{2+}]_i\) transients may depend on both chemical and gap-junctional transmission.

1.9 Neuronal migration in development

1.9.1 Radial and tangential migration of newly differentiated neurons

In the developing brain, the migration of NDCs from the site of their birth to their final destination is essential to proper circuit formation. Neuronal migration is a feature of all regions of the developing brain. However, three areas have received intensive study: the cerebral cortex, the cerebellar cortex and the rostral migratory stream (RMS - provides neuronal precursors to the olfactory bulb). The neurons of the cerebral cortex arise in the cortical VZ. Newborn neurons migrate to the outer edge of the cerebral wall to form the pre-plate, which at later stages in development expands to become the cortical plate. The intermediate zone (IZ) is located between the preplate and the VZ and consists of tangentially arranged fibres. Neurons take up their positions in the cortical plate in an inside-out sequence with newly arriving neurons migrating through the existing layers to the top of the plate. This radial migration was briefly mentioned in section 1.4.6. Radial glial cells span the entire thickness of the cortex and guide immature neurons to the correct location. Tangential migration is also a common feature of developing nervous systems. Lineage studies have shown that clonally related cells could end up located at great distances tangential to the point of their generation (Walsh and Cepko, 1993; Mione et al., 1994).

Granule cell PCs proliferate in the external granular layer (EGL) of the developing cerebellum. Following the final division, immature granule cell neurons move through the molecular layer (ML) and may further translocate through the Purkinje cell (PCL) and the internal granule layers (IGL). The mechanisms guiding the movement of cells through the ML appear to be similar to those that guide movement along radial glia fibres, in that cell adhesion molecules and other surface mediated interactions play
important roles (reviewed by Komuro and Rakic, 1998). However, much less has been known about migration in the absence of contact with glial fibres. Rakic and colleagues have addressed this with a series of studies involving confocal imaging of acute cerebellar brain slice culture systems (Komuro and Rakic, 1992, 1993, 1995, 1998; Rakic and Komuro, 1995). Granule cells undergo a series of changes in both shape and migratory behaviour as they traverse the ML and PCL. Their average rate of movement is fairly constant throughout the ML for animals of any given age, with the speed increasing at later times in development (9.6μm/h in 7 day old mice, to 18μm/h in 13 day old mice; Komuro and Rakic, 1995). However, whilst the average rate is constant, the movement is saltatory and is characterized by short stationary phases in between movements. Upon entering the PCL, the granule cell somata change from spindle-like to a more rounded shape. The rate of movement drops dramatically. After a prolonged stationary period in the PCL, the cells re-extend their processes, elongate their cell bodies and start to move again at an accelerated speed (Komuro and Rakic, 1998). These experiments demonstrated a consistent relationship between granule cell shape and the rate of movement, as well as showing that granule cells are able to undergo rapid radial migration in the absence of glial fibres to guide them.

1.9.2 Neurotransmitters and their receptors influence migration

The guidance cues regulating radial migration are unknown but several extracellular and cell-surface molecules have been implicated. Cell adhesion molecules and extracellular matrix cues may induce cells to change shape and their rate of migration (Rakic et al., 1994; for review is Frotscher, 1998). There is also increasing evidence of a role for ion channels and neurotransmitter molecules in modulating cell migration. Postmitotic granule cells start to express N-type Ca\(^\text{+}\) channels prior to radial migration to the ML and these appear to play an important role in the regulation of their migration (Komuro and Rakic, 1992). ω-conotoxin GVIA, a specific blocker of the N-type Ca\(^{2+}\) channel, greatly reduces cell migration in the ML in a dose-dependent manner. Interestingly, inhibition of other types of voltage-gated channels e.g. L- or T-type Ca\(^{2+}\) channels or Na\(^{+}\) and K\(^{+}\) channels failed to have any effect. Since activation of N-type Ca\(^{2+}\) channels causes significant increases in [Ca\(^{2+}\)]\(_{o}\) and speed up migration in granule cells, lowering the [Ca\(^{2+}\)]\(_{o}\) in a graded fashion should decrease the rate of migration by reducing Ca\(^{2+}\) entry. Komuro and Rakic (1992), through imaging of migrating neurons in mouse
cerebellar explant cultures, show that this is indeed so. Spontaneous changes in \([Ca^{2+}]_i\) are also observed during migration (see section 1.8.3 b) and these fluctuations correlate with periods of forward movement in the saltatory progress of these migrating cells (Komuro and Rakic, 1996). The rate of migration is positively correlated with both the frequency and amplitude of the transients.

The effects of VGCCs on granule cell migration suggest that neurotransmitter receptors may play a role in the regulation of movement. Activation of NMDA, AMPA/kainate, GABA_A and GABA_B receptors have all been shown to increase granule cell \([Ca^{2+}]_i\) (Connor et al., 1987; Howe et al., 1991; see also Laurie et al., 1992). The NMDA receptor appears to exert a powerful regulatory action on the regulation of these cells. Inhibition of NMDA receptors with D-AP5 or MK-801 reduces the rate of granule cell migration (Komuro and Rakic, 1993). In contrast, antagonists of AMPA/kainate, GABA_A or GABA_B receptors do not significantly affect their rate of movement (Komuro and Rakic, 1993). The presence of spontaneously active NMDA receptors on migrating granule cells has been confirmed in patch-clamp recordings in cerebellar slices (Rossi and Slater, 1993), with the frequency of spontaneous events increasing at the onset of migration. Furthermore, increasing extracellular glutamate levels by inhibiting glutamate uptake in surrounding astrocytes increased both the frequency of spontaneous NMDA-receptor induced activity and the rate of granule cell movement in the ML (Komuro and Rakic, 1993). Thus, glutamate, acting via NMDA receptors, exerts a key influence on granule cell migration.

It is well established that retinal PCs undergo INM (see section 1.4.2), translocating their nuclei in a manner similar to that seen in migrating neurons. NDCs born in the VZ also migrate to appropriate locations to form the laminar organization of the adult retina. Despite this, mechanisms that govern the INM of both PCs and the migration of NDCs in the retina have so far received little attention. Spontaneous \([Ca^{2+}]_i\) waves have been shown to play important roles in the formation of the retinal circuitry (see above) but whether other forms of spontaneous \([Ca^{2+}]_i\) activity, perhaps mediated by the activation of neurotransmitter receptors, play a role in regulating migration remains to be investigated.
1.10 Gap Junctions in the developing and adult retina

Gap junctions have been implicated in diverse roles in CNS development in processes including neurogenesis, apoptosis, synaptogenesis and pattern formation. Gap junctions are intercellular channels that permit electrical and chemical coupling between the cells of multicellular organisms. They are transmembrane complexes consisting of 12 subunits (connexin proteins, Cx), 6 of which form a connexon or hemichannel in the plasma membrane of one cell. The connexons of two adjacent cells form an aqueous pore with low electrical resistance, and a high permeability for small molecules (e.g. IP$_3$) and ions (e.g. Ca$^{2+}$) up to a molecular weight of ~1.5kD. The Cxs are a multigene protein family divided into three groups: α, β and γ. Each of these has different gating and conductance characteristics as well as distinct patterns of expression.

Gap junctions are found throughout the adult CNS (Dermietzel et al., 1989, 1993) and play important roles in the processing of visual information in the adult retina. They function as electrical synapses that may be modulated by a variety of neurotransmitters including dopamine and glutamate, as well as other molecules such as retinoic acid and Ca$^{2+}$. Interestingly, gap junctions are not directly responsible for transmission of visual information through the ‘vertical’ pathway. Rather, they act to mediate lateral connections within the plane of the retina, producing stereotypical coupling between cells of the same, rather than different, type (reviewed by Cook and Becker, 1995). In addition to the well-characterized homologous coupling between amacrine cells, bipolar cells and horizontal cells, heterologous coupling occurs between rods and cones and between bipolar cells and amacrine cells. The regulation of electrical coupling between retinal neurons in the adult appears to be an important part of the mechanism of ‘network’ light adaptation (Weiler et al., 2000; Mills and Massey, 1995). Adaptation allows the retina to function efficiently over a wide range of light intensities; changes in gap junctional coupling within the retina optimise retinal processing in accordance to the prevailing level of illumination.

The role of gap junctions in development is likely to be very different to that of electrical communication and spatial buffering of ions seen in the adult CNS. Gap junctions appear to have specific and transient developmental functions and have been implicated in many developmental events including neurogenesis (Becker and Mobbs,
1999), cell death (Lin et al., 1998), synaptogenesis (Allen and Warner, 1991) and coordinated activity required for the correct segregation of the central visual projections (see above).

There are distinct changes in the expression patterns of different connexins during development. In the adult rodent retina the expression of several Cxs has been described. mRNA for Cx 43, 32, 31, 26, 36, 37, 40 and 45 have been detected by PCR (Söhl et al., 1998) although immunoblots failed to detect the Cx26, Cx31, Cx32 and Cx40 proteins (for review, see Söhl et al., 2000). Positive immunostaining of Cx45 and Cx36 was detected in the IPL and OPL of the mouse retina and Cx43 was found in the GCL and is believed to be located on astrocytes. Connexin proteins are present in the retina from the earliest stages of development (Becker and Mobbs, 1999) and their expression is both temporally and spatially regulated. EM studies show that the presence of gap junctions declines with the progression of differentiation in the chick retina (Fujisawa et al., 1976). Antibodies raised against five different connexins in the chick showed that Cx43 is the first to be expressed throughout the eyecup (E4 onwards; Becker and Mobbs, 1999) and is temporally correlated with the first waves of cell proliferation. Its expression is then progressively restricted to the GCL and FL (Becker et al., 2002). Expression of Cx32 and Cx26 occurs from E7 onwards in the outer chick retina with Cx26 localized predominantly within the INL and Cx32 in the GCL and INL. Cx43 expression is similarly reduced in the developing hippocampus as cells differentiate and start to express other Cxs (Rozental et al., 2000). Expression of Cx36 by developing rat cortical neurons reaches a peak in the first two week of postnatal life, decreasing sharply by the third (Belluardo et al., 2000).

Extensive gap junctional coupling has been shown between neurons in the embryonic mammalian retina (Penn et al., 1994) and neocortex (Peinado et al., 1993a & b). In the VZ of the early mammalian neocortex, PCs and radial glia form gap junction-coupled clusters that extend radially through the VZ (LoTurco and Kriegstein, 1991b). This coupling may be specific to phases of the cell cycle (Bittman et al., 1997) and, following the final mitosis, a post-mitotic cell uncouples from the cluster before migrating away (LoTurco and Kriegstein, 1991; Bittman et al., 1997). It is conceivable that differentiated cells may re-couple to the columnar arrays (Peinado et al., 1993). Coupling between PCs and differentiated cells provides a direct pathway for the latter to
influence the fate of dividing cells. In cultures of dissociated RGCs cell-cell contacts are required to control, and restrict, the number of RGCs that differentiate (Waid and McLoon 1995). Becker and Mobbs (1999) demonstrated that down regulation of Cx43 using antisense oligodeoxynucleotides resulted in a reduction in the level of cell proliferation in the VZ suggesting that coupling is required for some part of the proliferative cell cycle.

Changes in gap junctional coupling are seen throughout development. For example, differentiating neurons in the cortical plate form extensive clusters. This coupling between differentiated neurons is down regulated at the time of synaptogenesis in both the cortex (e.g. Mienville et al., 1994; for review, see Rorig and Sutor, 1996) and the retina (Becker et al., 2002). A bell-shaped pattern of gap junction coupling is seen between developing rat cortical neurons in which a decrease in coupling parallels a dramatic increase in synaptic activity (reviewed by Kandler and Katz, 1995). Some of the changes in Cx expression occur over very short time periods. For example, Cx26 expression by chick starburst amacrine cells is seen for only 24h during a period when these cells may be involved in synaptogenesis (Becker et al., 2002).

1.11 The role of the RPE in neural retinal development

The RPE carries out a number of roles that are essential for the maintenance and proper functioning of the adult retina. In vision the RPE cells absorb stray light, preventing the scatter of light between photoreceptors. Another key role of these cells is to reduce all-trans-retinol, which is taken up from the photoreceptors, and transform it back to 11-cis-retinal. The 11-cis-retinal is then transported back to the photoreceptors. The RPE is also required for the phagocytosis of shed rod and cone outer segments, and various aspects of retinal nutrition. Studies of the albino eye (for review, see Jeffery, 1997) show that the RPE also plays an essential role in the normal development of the neural retina. Albino mammals lack a functional tyrosinase gene. Tyrosinase is an enzyme essential for the synthesis of melanin in the skin and the eyes. When melanin is absent or reduced, the retina develops abnormally. In albinos, many temporal retinal axons that would normally remain uncrossed at the optic chiasm cross inappropriately leading to innervation of the contralateral hemisphere. The central regions of the neural retina are underdeveloped, the INL and ONL being abnormally thin in these regions (Jeffery and
Kinsella, 1992; Ilia and Jeffery, 1996). Further, there is a rod deficit across the retina (Jeffery et al., 1994). Interestingly these abnormalities are not apparent in birds, which have cone-dominated retinas (Jeffery and Williams, 1994).

The maturation of the RPE is complete before that of the neural retina. In the chick, the RPE cells are born between E2.5 and E4 and the RPE becomes pigmented at around E4. In albino retinas, the maturation of the neural retina is delayed and the centrifugal gradient of differentiation is almost absent (for review, see Jeffery, 1997). There is evidence that dopa, an intermediate in the melanin synthetic pathway, can affect the cell cycle in the retina. Application of dopa to cultured RPE cells causes a lengthening of the cell cycle time from 19 to 27h (Akeo et al., 1994). In the neural retina the number of mitotic figures present in the VZ is markedly increased in albino animals compared with controls (Ilia and Jeffery, 1999). This leads to an abnormally thick retina, although this is only transitory as increased levels of cell death reduce the cell number to nearer normal levels. It has been suggested that dopa or another component in the melanin synthetic pathway acts to regulate the production of retinal cells by influencing cell cycle rates and exit points, and thus the developmental ‘window’ in which a given cell exits mitosis and undergoes differentiation (Ilia and Jeffery, 1999). The mechanisms by which dopa brings about these changes are unclear. However, a possible route is via gap junctional communication. There is evidence that gap junctions are present between the neural retina and the RPE during development (Hayes, 1976; Dixon and Cronley-Dillon, 1974). In the adult retina, many gap junctions are regulated by dopamine. It is possible that dopa plays a similar role in the developing retina, gating connections between the neural retina and the RPE.

Vollmer et al. (1986) showed that inclusion of RPE cells in retinal cultures led to a highly organized arrangement of differentiating cells that was similar to that seen in vivo. Rothermel et al. (1997) and Sheedlo and Turner (1996) demonstrated that RPE-conditioned media had similar effects on retinal explants, promoting proliferation, survival and differentiation of retinal PCs. These effects were absent if the RPE-conditioned medium was pre-treated with trypsin or heat suggesting the factor(s) involved is/are proteins (Sheedlo and Turner, 1996). One such factor is pigment epithelium-derived factor (PEDF), which was first identified as an RPE-derived protein with potent differentiating activity in human retinal cells (Tombran-Tink et al., 1991).
has now also been shown to have neurotrophic and neuroprotective roles in vivo (Cao et al., 1999; Jablonski et al., 2000).

Raymond and Jackson (1995) generated transgenic mice carrying an attenuated diphtheria toxin-A gene. This gene was driven by the promoter of a gene encoding the tyrosinase-related protein-1, which is specifically expressed in pigment cells. This caused the ablation of the RPE cells and produced eyes in which the laminar structure of the retina was completely disrupted. In some animals, a number of RPE cells escaped ablation and formed patches of pigmented cells. The neural retina immediately adjacent to such regions was of normal appearance but was disrupted elsewhere. These experiments clearly implicate the RPE in normal retinal organization although the signalling mechanisms and genes regulating this process are only just beginning to be discovered. Jensen et al., (2001) have provided further genetic evidence that signalling from the RPE to the retina is required for induction of correct retinal organization. They describe mutations of the mosaic eyes gene that cause RPE and retinal abnormalities including the loss of localisation of dividing cells to the VZ and loss of retinal lamination. Instead of dividing at the VZ before migrating to their correct laminar position, moe-mutant RPCs divide throughout the depth of the retina. They fail to migrate to the correct layers, settling in apparently random positions. This appears to affect late born cell types more than those born earlier in development such as the RGCs. The gene disrupted in moe therefore appears to be required for RPE cells to signal to the neural retina in order to establish the correct polarity of the retina. The RPE is a highly polarized epithelium. Moe may function to polarize the RPE itself, or may code for a factor that provides a polarity cue to the retina.

Recent experiments have also shown that ATP is released from RPE cells in a polarized manner, into the region adjacent to the neural retina, the sub retinal space (Mitchell, 2001). ATP is released from cultured human RPE cells in a biphasic manner following stimulation with growth factors (bFGF), pyrimidines (UTP) and on treatment with hypotonic solutions. Furthermore, RPE cells grown on permeable supports showed apical release of ATP, analogous to release into the sub retinal space in vivo. Purinergic receptors are present on the RPE; application of ATP or UTP causes a change in [Ca^{2+}], (Stalmans and Himpens, 1997). A number of other metabotropic receptors have been
identified on the RPE including those for dopamine, ACh, adrenaline and adenosine (Friedman et al., 1988; Dearry et al., 1990; Frambach et al., 1990).

### 1.12 Summary and aims of thesis

The production of cells by CNS progenitors and the determination of their fate are tightly regulated by a combination of genetic and environmental factors. For example, diffusible growth factors, such as FGFs, are believed to play key roles in the initial development of RGCs from the undifferentiated neuroepithelium (McCabe et al., 1999). Contact mediated interactions, such as those involving the transmembrane proteins notch and delta, play a critical role in the generation of neuronal diversity in the vertebrate retina (for review see Perron & Harris, 2000).

The role of neurotransmitters and synaptic transmission in the refinement of synaptic connections during development has been extensively studied (see section 1.8.3). However, there is growing evidence for the involvement of a variety of neurotransmitters in the regulation of earlier developmental events, some of which occur prior to synapse formation, such as proliferation, migration and differentiation. In order for these effects to be physiologically relevant, functional neurotransmitters and their receptors must be present early in development.

In the cortex ACh, acting via mAChRs, can stimulate PC proliferation or, under different experimental conditions, inhibit DNA synthesis (Baumgold and Dyer, 1994; Nicke et al., 1999; Li et al., 2001). Similarly, purine nucleotides and nucleosides can increase or decrease DNA synthesis in glia and neurons (Ciccarelli et al., 1994, Sugioka et al, 1999). GABA has also been reported to be able to both increase (Fiszman et al., 1999; Haydar et al., 2000) and decrease (LoTurco et al., 1995) cell proliferation, and to partially block the mitogenic actions of bFGF in the cortex (Antonopoulos et al., 1997). Like GABA, glutamate may also increase or decrease cell proliferation in the cortex by changing the cell cycle time; both glutamate and GABA increase the size of cortical VZ clones, while decreasing sub-ventricular zone clone size (Haydar et al., 2000). From these studies,
these studies, questions have arisen as to the mechanisms by which these transmitter systems exert their effects on cell division. Further, the same transmitter can cause opposing effects in different regions of the brain (e.g. Haydar et al., 2000). Whilst the retina is thought to use similar mechanisms of proliferation and differentiation as those used in the cortex, there is little data to support the notion of neurotransmitter-mediated control of retinal proliferation.

The primary aim of this thesis is the investigation of the effects of different neurotransmitters and their receptors on retinal development at a time prior to the formation of conventional synapses. Previous studies in the developing cortex have indicated that amino acid transmitters may act to slow down or speed up the progenitor cell cycle (LoTurco et al., 1995; Haydar et al., 2000). Further, there is circumstantial evidence to indicate that these transmitters may act via changes in $[\text{Ca}^{2+}]_{i}$ (LoTurco et al., 1995). In chapter 3 confocal imaging is employed to test whether or not spontaneous $[\text{Ca}^{2+}]_{i}$ events of a type similar to that seen in the neocortex (Owens and Kriegstein, 1998) are present during the early stages of retinal development. The VZ consists of both actively proliferating and newly differentiated cells. However, in previous experiments the identity of the cells demonstrating either spontaneous or evoked $\text{Ca}^{2+}$ events has rarely been determined. Here, dual imaging of $[\text{Ca}^{2+}]_{i}$ and chromatin will be used to establish whether or not actively proliferating cells and NDCs are capable of firstly, producing spontaneous $[\text{Ca}^{2+}]_{i}$ transients and secondly, responding to neurotransmitter stimulation. If PCs are capable of responding to such stimulation it may provide a mechanism by which endogenous neurotransmitters, released either by differentiated cells or by PCs themselves, could act in a paracrine manner to regulate the progenitor cell cycle.

In chapter 4, two protocols are used to test whether neurotransmitters can have direct effects on the proliferative cell cycle. The actions of muscarinic, purinergic, GABAergic and glutamatergic agonists and antagonists are assessed over two timescales. The first examines whether these reagents can affect the speed at which cells progress through mitosis. Mitosis is known to be tightly regulated and includes
several Ca\(^{2+}\)-dependent checkpoints (for review, see Whitaker and Larman, 2001). If neurotransmitters act by raising \([Ca^{2+}]_i\), it is possible that they may modulate the rate of progression through mitosis by influencing these checkpoints. Given that mitosis comprises only a short period of the cell cycle, the effects of chronic exposure to the same neurotransmitters are assessed. Such measures will give an indication of whether any potential actions of the neurotransmitters are either maintained, or compensated for, at other stages in the cell cycle.

The process of INM has received very little attention in developmental studies, yet is essential for two prominent periods of the cell cycle, those of \(G_1\) and \(G_2\). Mutations that disrupt INM lead to ectopic cell divisions and disrupted laminar organisation (e.g. Jensen et al., 2001) and thus it is important to establish the normal physiology of this process. A novel labelling technique is used to examine the process of INM in detail in chapter 5. Another form of cell movement, that of migration in immature neurons, has been studied extensively (Komuro and Rakic, 1993; 1995; 1996; 1998; Mione et al., 1994). These studies have revealed that neuronal migration is dependent upon changes in \([Ca^{2+}]_i\), and can be modulated by neurotransmitters. PC movements will be examined to determine if similar mechanisms are involved in INM. Specifically, are \([Ca^{2+}]_i\) transients correlated with movement and do neurotransmitters affect the speed of INM?

The final section of this thesis concerns another form of cell-cell communication; that of gap-junctional coupling. Gap-junctional coupling is high during early stages of development and is thought to be a requirement for proliferation in both the cortex (Bittman et al., 1997) and the retina (Becker and Mobbs, 1999). The anatomical pattern of connectivity is unknown. If PCs are directly coupled to NDCs it may provide a direct pathway for differentiated cells to influence those still proliferating. Alternatively, coupling between PCs may be a requirement for them to remain in the cell cycle. These possibilities are investigated in chapter 6. It has been suggested that gap junctional coupling may exist between the RPE and the underlying neural retina and that this provides a pathway for communication between these two tissues early in development. The presence of this hypothesised pathway is tested in chapter 6.
Later in chick retinal development, gap junction-dependent Ca$^{2+}$ waves sweep across large regions of the retina during synaptogenesis. Since gap junctions are present much earlier in development it is possible that similar events occur prior to this time. Confocal imaging is used in the first part of chapter 6 to characterise co-ordinated Ca$^{2+}$ activity occurring in the first week of development and to determine whether these events are dependent upon gap junctions. This will be necessary for future investigations into the role of such activity early in retinal development.
Figure 1.1 Anatomy of the mature chick retina. The mature chick retina comprises 6 types of neuron and 1 type of glial cell, the Müller cells (MC). Photoreceptors (PR), of which there are two types, rods and cones, bipolar cells (BC) and ganglion cells (GC) constitute the vertical pathway for information flow through the retina. Action potentials are conveyed to the brain by the ganglion cell fibres (FL). Horizontal cells (HC) and amacrine cells (AC) modulate the vertical pathway at the level of PR-BC and BC-GC synapses, respectively. All these cell types originate from a single cell thick neuroepithelium through the tightly orchestrated proliferation and differentiation of pluripotential progenitors. PRL: photoreceptor layer; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.
Figure 1.2 Mitosis. A, The cell cycle is divided into four distinct phases: Mitosis and cell division occur during the relatively short M-Phase. This is followed by the G\textsubscript{1} (gap) phase, which may often cover the longest part of the cycle. G\textsubscript{1} separates M-Phase from S-Phase during which DNA replication takes place. During G\textsubscript{1}, the cell (with duplicated DNA) prepares for mitosis. Terminally differentiated cells assume a quiescent state called the G\textsubscript{0} phase. B, The different stages of mitosis (M-phase), from prophase to cytokinesis, are characterized by specific changes in the arrangement of the chromosomes (shown in green). The most conspicuous of these, as seen with Hoechst staining, are the alignment of the chromosomes during metaphase and their segregation during anaphase/telophase.
Figure 1.3. Interkinetic nuclear migration. A, PCs undergo INM. In this process the PC nucleus moves between the VZ, which is permissive for mitosis (M), and the GCL. The nucleus moves toward the GCL in $G_1$, replicates its DNA (S-phase) and returns toward the VZ in $G_2$. Throughout this period the cell retains contact with both surfaces of the retina by means of thin cytoplasmic processes. During the transition from $G_2$ to M-phase the cell retracts its vitreal process before dividing. PCs can either undergo symmetrical division in which both daughter cells continue in the cell cycle, or divide asymmetrically, to give rise to a PC and an NDC that then migrates to its final location. B, Combined phase-contrast and fluorescence image of a section through an embryonic day (E)6 chick retina, stained with propidium iodide (red). Mitotic cells are confined to the VZ, immediately adjacent to the RPE. Mitotic cells can be identified by the presence of strongly staining, and highly condensed, chromatin that contrasts with the weakly stained and dispersed chromatin of the interphase cells. C, Single PC injected with FITC-dextran (green) to show the processes that extend to the ventricular and vitreal surfaces. The nucleus of the cell is located in the bulge within the cell below the VZ. Scale bars 5μm NB. Propidium iodide is a red-fluorescent nuclear and chromosome counterstain.
Figure 1.4 Cell birth in the chick retina based on $^3$H-thymidine labelling studies (Kahn, 1974; Prada et al., 1991). Neural development involves two main phases: proliferation and differentiation. Differentiation occurs in several overlapping waves, with RGCs emerging earlier than any other cell type followed by photoreceptors, horizontal, amacrine, bipolar and finally Müller cells.
Figure 1.5 A, Symmetric and asymmetric cell division. A symmetric non-terminating division occurs in the plane of the epithelium and results in two daughter cells that have progenitor-like behaviour and morphology. An asymmetric division occurs perpendicular to the plane of the epithelium. The apical cell retains progenitor-like behaviour whilst the basal cell differentiates. Terminating, symmetrical divisions take place in the plane of the epithelium but result in two daughter cells that exit the proliferative cell cycle and differentiate. B, Metaphase rotations. Schematic diagram viewed as if looking down on the VZ of the cortical surface (an ‘xy’ view). Metaphase plates appear as rods when undergoing symmetrical division. Prior to entering anaphase the rods rotate (times 0-3). Very occasionally the plates ‘flip’ to an asymmetric orientation, as is shown at time point 4, before returning to the rod orientation (time 5).
Figure 1.6 Neurotransmitter receptor expression in the developing retina. Diagram is based on the $[Ca^{2+}]_i$ responses evoked by neurotransmitters in whole chick retina (Purinergic: Sugioka et al., 1996; Sakaki et al., 1996; Muscarinic: Yamashita et al., 1994; Sakaki et al., 1996; Allcorn, 1996; GABAergic: Yamashita and Fukuda, 1993; Allcorn, 1996; Catsicas and Mobbs, 2001; Glutamatergic: Allcorn, 1996; Sugioka et al., 1998). Key: P2Y-R=purinergic receptor, mAChR=muscarinic ACh receptor, nAChR=nicotinic ACh receptor, NMDA-R and AMPA/KA-R=glutamate receptors, GABA$_A$-R and GABA$_B$-R=GABA receptors. Arrowhead indicates latest date studied. Muscarinic, purinergic and GABA$_A$ receptor-mediated $[Ca^{2+}]_i$ responses have been shown to be absent at the ages indicated.
Chapter 2

Methods

2.1 Preparation of retinal tissue

White Leghorn chicken eggs were incubated at 36°C and 60% relative humidity. Chicken embryos between E4 and E6 were killed by decapitation according to Schedule 1 of the Home Office Regulations (1986), and the eyes removed. Embryos were staged using the method of Hamilton and Hamburger (1951). E4 corresponds with stages 23-25, E5 with stages 26-28 and E6 with stages 29-30. Animals not fitting within the appropriate range were rejected.

Retinae were dissected from the eyecup at room temperature (RT) in Krebs’ solution containing (in mM): 100 NaCl, 30 NaHCO₃, 6 KCl, 1 MgCl₂, 3 NaH₂PO₄, 1 CaCl₂ and 20 Glucose (pH 7.4 by gassing with 5% CO₂/95% O₂). A cut was made around the eye at the level of the ora serrata, the lens and sclera were removed and the RPE peeled away from the neural retina. Any remaining vitreous humour was carefully removed. Experiments involving the imaging or patch clamping of cells in the RPE (Chapters 4 & 6) required the removal of the overlying sclera whilst leaving the RPE intact. In order to minimise the disruption of connections between the RPE and the neural retina through mechanical stress, the RPE’s attachment to the overlying sclera was weakened enzymatically by pre-incubation with 0.05% dispase for 30mins, followed by careful dissection.

2.2 Superfusion system

Extracellular solutions were bath applied in all imaging experiments. A gravity-fed constant-level perfusion chamber, supplied by a peristaltic pump, allowed the superfusion of the retinae (Figure 2.1) with gassed Krebs’ solution at a constant rate of 0.5ml/s and the rapid exchange of solution in the perfusion chamber (<3s). Drugs were introduced by moving the pipe supplying the peristaltic pump from a reservoir containing control solution to one containing the appropriate drug. Washout periods
varied according to the drugs used but were always significantly larger (>15mins) than the time taken for apparent recovery.

2.3 Preparation of drugs

The following drugs were made as stock solutions in distilled water at either 10mM or 100mM final concentration (depending on solubility): ACh (acetylcholine chloride), CCh (carbachol), muscarine, nicotine, pirenzipine, UTP (uridine 5'-triphosphate), UDP (uridine 5'-diphosphate), ATP (adenosine 5'-triphosphate), ADP (adenosine 5'-diphosphate) suramin, PPADS (Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt), Reactive Blue (1-Amino-4-[[4-[[4-chloro-6-[[3 (or 4)-sulfophenyl]amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenyl]amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid), caffeine, nickel chloride, GABA (γ-aminobutyric acid), bicuculline, glutamate (all Sigma, England), AMPA ((±)-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid), NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide), D-AP5 (all Tocris, England). AFDX-116 (Otenzepad/1 l-[[(Diethylamino) methyl]-1-piperidinyl] acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one, Tocris) was made up to 10mM in DMSO (Dimethyl sulphoxide, Sigma) and tropicamide (Sigma) was made up to 10mM in ethanol. Drugs were then diluted appropriately. Where experiments involved drugs made up in DMSO or ethanol, these solvents were included in the control solution at the appropriate concentration.

2.4 Ca\(^{2+}\) imaging

2.4.1 Loading of retinae with Ca\(^{2+}\)-sensitive dyes

After dissection, retinae were loaded with Ca\(^{2+}\)-sensitive dye by immersion in 1ml of Krebs' solution containing Fluo-4 AM (10μM, Molecular Probes, England) and the dispersant Cremophor-EL (0.03%, Sigma) for 1h at RT\(^*\) (for experiments to determine responses to application of exogenous agonists) or at 36°C (for experiments to monitor spontaneous activity). 10mins prior to the end of Fluo-4 loading, the vital chromatin dye Hoechst 33342 (2μM, Molecular Probes) was added to the loading solution. After loading, the retinae were maintained in Krebs' at RT or 36°C (as above) for up to 2h

* Spontaneous activity is rare at RT and therefore does not interfere with agonist-induced responses.
before use. All loading was carried out in a dark chamber in an atmosphere of 95% O2/5% CO2.

For confocal imaging the central piece of the retina (~5x5mm, or the whole retina for E4 preparations) was placed in a temperature controlled, glass-bottomed perfusion chamber (1ml volume) on the stage of an inverted microscope (LSM 510, Zeiss, Germany) and held in place with a platinum wire harp strung with a grid of parallel nylon threads. The U-shaped frame (10x10mm) of the grid was made from 1mm platinum wire, flattened with a vice (Konnerth et al., 1987), and threads taken from nylon tights were glued to the frame. The recording chamber was perfused with gassed Krebs' solution and held at either RT (22°C) or at 36°C (see above) using a thermostatically controlled heating element (see Figure 2.1).

2.4.2 Imaging data acquisition, storage and analysis

With the exception of preparations used in ‘virtual z scans’ (see below), retinæ were imaged as flat mounts, with the VZ facing the objective (unless otherwise stated). Experiments were conducted at RT or 36°C (see above). The fluorescence of Fluo-4 and of Hoechst 33342 was excited with the 488nm line of the argon laser and the 350nm line of the UV laser, respectively. Inclusion of centre-to-periphery developmental changes was avoided by imaging the central region of each retina. Single Hoechst images were taken immediately prior to, and after, drug application to monitor the state of the chromatin and the stability of the preparation during recording. Fluo-4 images were acquired at 4s intervals (unless otherwise stated) and analysed offline using Lucida 5.0 or 6.0 software (Kinetic Imaging Ltd., Liverpool, England). The mean fluorescence of individual cells was measured and normalized to its initial value at time 0. Traces are plotted as the change in fluorescence intensity (ΔF) expressed as a percentage of the fluorescence (F) observed at the start of the recording, in the absence of stimulus, thus:

$$\frac{\Delta F}{F} = \frac{(F - F_{\text{rest}})}{F_{\text{rest}}}.$$  

A change in fluorescence in excess of a criterion level of 10% above baseline was accepted as a response. These criteria were established following the observation of several control preparations. The value of 10% included all spontaneous [Ca2+]i, events
observed visually and appeared to prevent the inclusion of fluctuations in fluorescence that may occur due to slight changes in focus. It is possible that some smaller events were missed using this criterion and thus changes in activity may incorporate a small under-estimation. Data are presented as means ± standard error of the mean (SEM). Unless otherwise stated, all data concerning spontaneous and evoked changes in $[\text{Ca}^{2+}]_i$ were normally distributed, as determined using the Kolgomorov Smirnov test (GraphPad Prism 3.00) and statistical significance was assessed using an unpaired Student’s t-test. The biggest limitation of the techniques used here is the relatively low temporal resolution which will mean that fast events (<1s duration) may be missed.

Experiments are described in Chapters 3, 5 and 6 that involve the measurement of changes in $[\text{Ca}^{2+}]_i$ in cells throughout the depth of the retina. Whilst the z-line scan function available on the Zeiss LSM 510 provided acceptable assessment of changes in $[\text{Ca}^{2+}]_i$ over a short time-scale (e.g. during the application of an agonist), this technique was not suitable for imaging over longer periods (>2mins) for two reasons. Firstly, even the smallest lateral movement caused a change in the line section imaged such that fine processes could not be relocated. Second, the laser used for the line scan function caused very significant dye-fade at the laser powers required to produce an image of adequate resolution. Therefore, an alternative technique was developed in which Fluo-4 AM loaded retinae were deliberately folded before being held underneath a harp. Imaging the folds in the xy plane creates a ‘virtual z section’. Such folded preparations allowed the use of an xy scan at a single focus level, and included all layers of the neural retina (and RPE where appropriate). Retinae were imaged as described above and analysed off-line.

2.4.3 Calibration of $[\text{Ca}^{2+}]_i$

The excitation and emission spectra of Hoechst 33342 and Fluo-4 allow the complete separation of the signals from the DNA and Ca$^{2+}$ changes within the cell. Of the Ca$^{2+}$-sensitive dyes with emission spectra in the green regions of the spectrum, Fluo-4 is one of the most fade-resistant and delivers a high quantum yield, and is therefore suitable for imaging over extended periods. However, it is not a ratiometric dye. Searches failed to discover any ratiometric Ca$^{2+}$ indicator that had both suitable spectral characteristics for simultaneous use with Hoechst 33342 and that was sufficiently resistant to photobleaching. In order to quantify the fluorescence signal from Fluo-4 it
is necessary to determine the maximum and minimum values of fluorescence by artificially altering \([Ca^{2+}]_i\) to saturating and zero levels. These values can then be inserted into the equation:

\[
[Ca^{2+}]_i = KD \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}
\]

where the \textit{in vitro} KD of Fluo-4 for \(Ca^{2+}\) is 345nM (Molecular Probes).

In order to calibrate the dye, retinae were loaded with Fluo-4, as described above, and transferred to the stage of a confocal microscope. Control solution was exchanged for one containing high \([K^+]\) and zero \(Ca^{2+}\) containing (in mM): 50NaCl, 56 KCl, 30 NaHCO₃, 2 MgCl₂, 1 NaH₂PO₄, 1 NMDG EGTA and 20 Glucose. Single x4 line-averaged images were taken at the start of recording, in control Krebs', and at 2min intervals after a 10min delay following the exchange to high \([K^+]\) solution, until no further decrease in fluorescence was detected. This was taken as \(F_{\text{min}}\). For determination of \(F_{\text{max}}\) retinae were washed in normal Krebs' solution and transferred to Krebs' containing 0.1% Triton X-100 (Sigma). Images were taken at 2min intervals until no further increase in fluorescence was detected. Because of dye fading and the tissue distortion and swelling induced by treatment with high \([K^+]\) and Triton-X-100, it was not possible to routinely calibrate the dye using this procedure. To provide an indication of the resting mean \([Ca^{2+}]_i\) and that evoked by UTP and CCh (both 50μM) retinae were split in two. One half was used to determine \(F_{\text{min}}\) and \(F_{\text{max}}\) and the other half to determine the changes in fluorescence evoked by the drugs. Average values of the peak fluorescence following application of each agonist were obtained from 20 cells. The average resting \([Ca^{2+}]_i\) at the start of recording, after background correction, was calculated to be 73±9nM. The mean change in \([Ca^{2+}]_i\) following application of 50μM UTP was 687±32nM (range 218-1209nM, n=16). A different region was imaged during the application of 50μM CCh. The average resting \([Ca^{2+}]_i\) at the start of recording was calculated to be 71±9nM. The mean change in \([Ca^{2+}]_i\) following application of 50μM CCh was 587±66nM (range 278-1039nM, n=17).

Routine calibration of \([Ca^{2+}]_i\) in intact retinae was impossible (see above) and so, in common with others working with brain slices and retinal preparations (Nadal et al., 1998; Sugioka et al., 1996; for review see Takahashi et al., 1999a), \(\Delta F/F\) measurements were not routinely converted into \([Ca^{2+}]_i\) concentration. Apart from
during dye saturation, \( \Delta F/F \) is thought to reflect [Ca\(^{2+}\)]; if there is no significant change in dye concentration or other factors such as [pH], (see Takahashi et al., 1999a).

2.4.4 *Ratiometric measurements show that changes in [Ca\(^{2+}\)], are faithfully reflected by changes in Fluo-4 fluorescence intensity.*

Since PCs move between the VZ and vitreal surfaces during the proliferative cell cycle, it is possible that transient changes in Fluo-4 intensity seen during migration or other cell movements are due to changes in the volume of the cell within the imaging plane, rather than changes in [Ca\(^{2+}\)]. Therefore, ratiometric measurements were carried out to confirm that changes in Fluo-4 fluorescence accurately represent [Ca\(^{2+}\)] levels. Retinae were co-loaded with Fluo-4 AM (10µM) and Indo-1 AM (5µM) and the dispersant Cremophor-EL (0.03%, Sigma) in normal Krebs’ for 1h at 36°C. Indo-1 and Fluo-4 were excited using the 350nm line of the UV laser, and the 488nm line of the argon laser, respectively. Fluorescence emission from Fluo-4 and Indo-1 was recorded simultaneously at 405±10nm and 480±10nm, respectively. Upon binding Ca\(^{2+}\), Fluo-4 exhibits an increase in fluorescence, whilst the fluorescence of Indo-1 AM emission collected at 480±10nm decreases upon binding Ca\(^{2+}\) (Molecular Probes). Therefore, the ratio between Fluo-4 and Indo-1 fluorescence reflects changes in [Ca\(^{2+}\)]. Uniform changes in the fluorescence of both dyes are expected if the volume of a cell in the imaging plane changes or a more brightly labelled part of the cytoplasm enters the confocal plane. Dual emission images of VZ cells or of migrating cells in ‘virtual z-sections’ in a single confocal plane were collected every 5-10s. Fluo-4/Indo-1 ratio images were calculated by dividing the pixel intensity values for Fluo-4 by those for the same pixels when Indo-1 was excited. Figure 2.2 shows an example of fluorescence changes occurring in cells in the xy plane of the VZ, and another in a migrating cell within a ‘virtual z section’. Simultaneous dual-emission images revealed that Fluo-4 and Indo-1 co-localized within cells. Transient increases in the fluorescence of Fluo-4 were always accompanied by decreases in the fluorescence of Indo-1. Because of its relatively poor quantum yield, the fluorescent changes in Indo-1 were smaller than those of Fluo-4, but the onset, shape and time course of the [Ca\(^{2+}\)] changes obtained with the Fluo-4/Indo-1 ratio were the same as those obtained with Fluo-4 alone. These results confirm that changes in [Ca\(^{2+}\)], are faithfully reported by changes in Fluo-4 fluorescence alone. Since Indo-1 fluorescence fades very rapidly,
by comparison with that of Fluo-4, and because its excitation and emission spectra are similar to those of Hoechst 33342, it was not possible to use Indo-1 together with Fluo-4 for the long experiments described in this thesis. For these reasons, and those discussed in 2.4.3, changes in Fluo-4 fluorescence, and hence $[\text{Ca}^{2+}]_i$, are presented as $\Delta F/F$.

2.5 Real-time imaging of mitosis

2.5.1 Tissue preparation

E5 chick retinas were dissected out, as described above, and kept in Krebs' solution, at 36°C. Retinas were dissected as required and kept for no longer than 90mins prior to use. For each embryo, one eye was 'treated' with a drug and the other served as the 'untreated' control. The second eye was maintained at 36°C with RPE and lens in place, but with the sclera removed, until dissection immediately prior to the experiment. Ten minutes prior to the use of any retina, 2μM Hoechst 33342 was added to the bathing solution.

2.5.2 Imaging data acquisition, storage and analysis

Retinas were imaged as flat mounts with the VZ facing the objective. xy images of the Hoechst-stained chromatin in the VZ were acquired at 15s intervals for 90mins before being analysed off-line. The laser power was reduced to a minimum (<1%) in order to reduce any photo damage. The bathing solution was kept at 36°C throughout. Time-lapse movies of mitosis in the VZ were analysed using Lucida 5.0 or 6.0 software. In experiments that estimate the effects of transmitters on the rate of mitosis, the time spent in metaphase was measured. This period was defined as the time from which condensed chromatin is first seen as a rod-like structure to the first separation of the chromosomes (Figure 2.3). These transitions were distinct and occurred within 1-3 images. Whilst every effort was made to use the contralateral eyes as controls, this was not always possible due to the technical difficulties of maintaining stable recordings and, in the appropriate experiments, the fragile attachment between the RPE and the neural retina for such long durations. Therefore, data were pooled to give an average time within each test group and compared against the respective control group. Results
are given as means ± SEM. All data concerning the speed of mitosis were normally
distributed, as determined using the Kolmogorov Smirnov test (GraphPad Prism 3.00)
and statistical significance was assessed using an unpaired Student's t-test. Given that
the measurement of the length of time cells spent in metaphase involves quantitative
assessment where subjective bias could influence results, these movies were analysed
“blind” after the recordings were re-coded prior to analysis by a person not involved in
the experiments.

Metaphase plates with a rod-like appearance constantly rotate within the plane of the
VZ around a central axis, the rotations ceasing upon entering anaphase. In section
4.3.2, quantitative analysis of the rotational movements made by the mitotic apparatus
was performed on a sample of cells imaged for the experiments described above.
Measurements were taken from every 5th image in a movie. The orientation of a
metaphase rod at time point \( n \) was measured relative to the mean orientation adopted
by the chromatin of that cell during anaphase. The orientation of the mitotic apparatus
during metaphase was measured by drawing a line along the longitudinal axis of the
bar of stained chromatin (metaphase rod) of a dividing cell (red line in Figure 2.3).
The orientation at anaphase was determined by drawing a line parallel to the two
parting clusters of chromatin (blue line, Figure 2.3). For each cell, the average of all
measurements taken during anaphase was calculated (green line, Figure 2.3). The
angle measured between the mean orientation at anaphase and the orientation of the
metaphase rod at each time point was normalized, relative to the mean anaphase angle
(0°). Thus, 0° corresponds to an orientation that is parallel, and 180° corresponds to an
orientation that is anti-parallel, to the mean angle at anaphase. Time sequences are
presented as polar plots of orientations versus time. The measurements were then
sorted into 30° bins and plotted as “rose diagrams”, where the area of each sector is
proportional to the time spent at the angles within the bin.

In order to compare the orientations of the mitotic apparatus during metaphase, relative
to the angle adopted at anaphase, all observations from a population of 20 cells were
sorted into those occurring during metaphase, and those occurring after the onset of
anaphase. These measurements were sorted into 10° bins and plotted as rose diagrams.
The mean vector, vector length (\( r \)) and Rayleigh’s coefficient were calculated for the
distribution of angles at metaphase and at anaphase. The data was accurately described

72
by a Von Mises distribution (a unimodal model, tested using Oriana software for Windows, version 1.06) and so the Watson's F-test for two circular means was used to determine whether or not the mean vector lengths were significantly different.

2.6 Experiments to determine the effects of drugs in ovo

2.6.1 Application of drugs in ovo

Incubated eggs were "windowed" at E5 by cutting a 1cm² hole in the shell and the inner membranes were opened. Agonists (final concentration in the egg ~50μM) and antagonists (final concentration ~25μM) were injected (125μl total volume) into the amniotic pouch, using a micropipette, close to the heart of the embryo. Controls received sham injections of phosphate buffered saline (PBS). It was not possible to inject drugs directly into the eyes at E5 without causing severe disruption to their normal development (data not shown). Furthermore, the blood-retina barrier (BRB) is not complete at this age (Kneisel and Wolburg, 1993). Eggs were resealed with Sellotape and incubated for 8h at 36°C. Embryos were fixed with paraformaldehyde (PFA, TAAB Laboratories Equipment Ltd., Reading, UK) at a concentration of 4% in PBS applied in ovo, until the heart stopped beating, and maintained in vitro in PFA for a further 6h at 4°C. Within-embryo controls cannot be obtained when drugs are applied in ovo and, therefore, control data was obtained from embryos that were set to develop at the same time as those subject to drug applications. Whilst full staging was not possible prior to drug-application, the windowed shells enabled a visual assessment of all control and drug-treated embryos, in ovo, and ensured that they were at a similar stage in development prior to use (Hamilton and Hamburger scale, as described above). Following fixation, body length was measured from the base of the head to the tail tip. The eye diameter was measured through the choroid fissure (Figure 2.4a) using low power images taken using a CCD camera. Control data was pooled and the mean eye diameter for these embryos calculated. The eye diameter of each individual control or drug-treated embryo was then expressed as a fraction of that of the mean control value.
2.6.2 Preparation of retinal sections

For sectioning, the eyes were rinsed 3 times with PBS before being placed in 20% sucrose/PBS overnight at 4°C. The eyes were then embedded in Tissue-Tek O.C.T. (Sakura Finetek Europe B.V., The Netherlands) and frozen onto the chuck of a cryostat microtome (2800 Frigocut-E, Leica, England). Transverse retinal sections 20µm thick were prepared and affixed to poly-L-lysine coated slides. Every 3rd section was collected. Sections passing through the centre of the retina, adjacent to the optic nerve were processed as follows: Hoechst 33342 (2µM in PBS) was applied to sections for 5mins, in the dark, and then washed for a further 5mins in PBS before mounting in Citifluor (Citifluor Ltd, England). Hoechst fluorescence was detected using a confocal microscope, as described above. A pseudo-random sampling technique was used. Ten regions, approximately equally spaced around the retina, from each section were imaged. This procedure was repeated on three retinal sections from the eye of each embryo.

2.6.3 Data analysis

Mitotic cells were identified from their condensed chromatin and included all stages from prophase to telophase. The total number of mitotic cells in the VZ of each imaged region was counted and the mean number per 100µm length of retina was calculated for each embryo. Results are expressed as the percentage difference compared to controls. Pyknotic cells appeared as small, densely stained profiles. The same sections were used to determine the mean number of pyknotic nuclei per 100µm of retina. Data concerning eye size, and mitotic and pyknotic profiles within the VZ were normally distributed as determined using the Kolmogorov-Smirnov test (Graphpad Prism 3.00). Since more than one experimental group (agonist and antagonist) was assessed against the same control group statistical significance was determined using ANOVA and Dunnett’s correction for multiple comparisons against a single control.

Dividing cells were also measured to determine whether they were dividing vertically or horizontally with respect to the plane of the retina. All cells in each imaged region that could be clearly identified as being in metaphase, anaphase or telophase with a plane of cleavage perpendicular to the plane of the tissue section (i.e. the metaphase
plate looked like a 'rod' rather than a 'disk') were analysed. The angle of the metaphase 'rod' was defined as the acute angle, $\alpha$, between lines 2 and 3 as shown in Figure 2.4b. Line 1 is a line parallel to the plane of the RPE. Line 2 is a line perpendicular to the RPE and passes through the centre of the metaphase rod. Line 3 is a line passing through the length of the rod. An angle of 90° corresponds to a division in which the metaphase rod is at right angles to the plane of the RPE (a vertical division). Conversely, a division in which the metaphase rod is aligned parallel to the RPE (a horizontal division) has an angle of 0°. The data from each embryo was sorted into 10° bins and each bin expressed as the mean percentage ± SEM. This was repeated for all embryos in each agonist, antagonist and control group. The distributions of angles of division did not fit a normal distribution. Therefore, the Kruskal Wallis rank test was applied to determine the effect of agonists and antagonists on the distributions of angles. The same data set was used to calculate the proportion of 'vertical' versus 'horizontal' divisions, where 'vertical' and 'horizontal' were defined as cells dividing at $\geq 45°$ and $< 45°$ respectively. Statistical differences in the proportions of cells dividing vertically or horizontally were assessed using the Mann Whitney-U test.

Cell density counts were carried out on each eye by randomly selecting three 5000\( \mu \)m\(^2\) regions from at least three non-adjacent sections near the optic nerve for each embryo. The number of Hoechst-stained nuclei within each area was counted and a mean ± SEM was determined for each embryo before being compared against controls using ANOVA.

All cell counts and measurements of the angle of cell division were analysed "blind". Slides and images were re-coded prior to analysis by a person not involved in the experiments.

2.7 In vivo dye labelling of retinal cells using a biolistic technique

The biolistic dye-labelling technique has been adapted from a protocol used by Gan et al. (2000). Gold or tungsten particles are coated with dyes and fired into retinal tissue by means of a hand-held Helios gene gun (Biorad, UK). This allows for
multiple, but sparsely distributed, cells to be labelled in a living preparation and the
real-time imaging of INM and process extension and retraction.

2.7.1 Coating biolistic particles with lipophilic dyes

Polyvinylpyrrolidone (PVP, Sigma) was made up in ethanol at a concentration of 100
mg/ml before being poured into tubing (Biorad, UK). The tubing was inserted into a
tubing preparation station (Biorad), which rotates the tube to ensure its even coating,
and gasses it simultaneously with nitrogen to dry the interior. Stock solutions of the
carbocyanine membrane dyes, Dil and DiO (Molecular Probes) were prepared by
dissolving either 1mg Dil, 1mg DiO or a 0.5/0.5mg mix of the two in 100μl
dichloromethane (Sigma). 50mg of tungsten particles (0.7μm diameter, Bio-Rad),
which were found to be as effective and much cheaper than gold particles, were mixed
with 20μl of carbocyanine dye solution and spread on to a glass slide. The particles
were then left until completely dry (~20mins). As the liquid evaporates off, the dye
precipitates on to the tungsten particles. The dye-coated particles were removed from
the slide by scraping with a razor blade before pouring them into ~25cm length of pre­
coated tubing and distributing them by shaking. Gene gun “cartridges” coated with
these “bullets” were prepared by cutting into appropriate lengths ready for firing.

2.7.2 Particle delivery

Retinae were dissected as described above and the RPE removed, before being
flattened on a slide and removing any excess solution. Dye-coated particles were
delivered to the preparation using the gene gun (Figure 2.5). Track-etched membrane
filters (cell culture inserts, Falcon, Becton Dickinson Labware) with a 3μm pore size
and 8.0×10^5 pores/cm^2 were inserted between the gun and the preparation to prevent
large clusters of particles from landing on the tissue. The membrane filter also appears
to protect the tissue from the shock wave generated by the gun at high pressure. The
particles were fired at 70-80psi with the gun nozzle as close to the tissue as the filter
permitted (~2cm). Altering the distance between the filter and the tissue controlled the
density of particles hitting the tissue, and thus the number of cells labelled.
2.7.3 Imaging and data analysis

Labelled retinas were imaged on an inverted confocal microscope (either a Zeiss LSM510 or a Leica SP2). The specimens were held flat using a platinum harp, as described previously, and constantly perfused with Krebs solution and held at 36°C. Labelled cells were located using epi-fluorescence illumination and then imaged using either the 568nm line of the argon-krypton laser for DiI, or the 488nm line of the argon laser for DiO. x63 magnification images of the xy plane of the retina were taken at 1.0 μm steps, throughout the depth of the retina and ~5μm either side. This was repeated at 15min intervals. Images were analysed off-line using Metamorph software. The individual xy scans were built into a xyz stack to give a 3D image of the retina (see Figure 2.6 for detail). Individual image stacks were rotated about the x-axis and projected to give an xz view. This was repeated for each time point and the final images built into an xz projection ‘movie’. Measurements of the position of cell nuclei were made relative to the ventricular surface. The centre of the cell body was defined as the intersection of two diagonal lines drawn between the corners of a box fitted to the bulge containing the cell nucleus (Figure 2.7). The distance between this point and the outer edge of the VZ was measured using either Metamorph or Zeiss Image Examiner software. Measurements were made for each time point and the speed of migration determined. The length/width ratio of the cell body was determined manually with mouse-driven imaging software (Kinetic).

In experiments that examined the [Ca^{2+}]i transients in migrating PCs, E5 retinas were dissected as described before and loaded with Fluo-4 and imaged on a confocal microscope and perfused with Krebs’ held at 36°C. The retinas were deliberately folded and imaged as ‘virtual z sections’ (see section 2.4.2). Images were taken at 10 or 40s intervals with x2 line averaging and analysed off-line using Lucida software.

The distribution of the speeds of INM was skewed. In order to perform parametric analysis, data was transformed to a Lognormal distribution by taking the log 10 of all speeds (GraphPad Prism 3.00). The speed of INM in cells moving towards and away from the VZ were compared with an unpaired Student’s t-test. In experiments examining the effects of drugs on INM, data from drug-treated eyes was compared
with that from the contralateral control eye using a paired Student’s t-test. The relevant results were transformed back to the original scale.

2.8 Dye-injections and determination of dye-coupling

The pattern of dye coupling between retinal PCs, RPE cells, and between RPE cells and the neural retina, was investigated by whole-cell patch-clamping of cells using pipettes filled with a mixture of fluorescein-dextran (MW 3,000, Molecular Probes) and Neurobiotin (MW 323, Molecular Probes). The low molecular weight Neurobiotin is able to diffuse through gap-junctions, and therefore into any coupled cells. The high molecular weight of the fluorescein-dextran ensures it stays trapped within and provides a marker for the injected cell. After histochemical treatment to reveal the Neurobiotin (see below), the tissue was examined on a confocal microscope.

2.8.1 Dye filling using whole cell patch clamping

E5 retinae were dissected out, either with or without RPE in place (see section 2.1). They were held in place under a harp, with the ventricular side uppermost, in a perfusion chamber and constantly superfused with Krebs’ solution at 36°C.

Glass pipettes were pulled from thick-walled borosilicate glass with internal filaments (No. GC150F10, Clark Electrochemical Instruments) on a Mechanex BBCH puller. The tips of the pipettes were filled by capillary action with a dye solution containing 1% FITC-dextran/1% neurobiotin in an internal solution containing (mM): 103 Cs-glucocante, 2 MgCl₂, 0.1 CaCl₂, 40 HEPES, 5 NMDG-EGTA, 1 Na₂ATP (pH 7.35 with CsOH). This internal solution was chosen because experience has shown that it gives better preservation of cell structure (Becker et al., 2002). Target cells for whole-cell recording were identified using a microscope equipped with DIC optics and a UV illumination system.

The pipette and head-stage were mounted on a Narashige micromanipulator and moved under visual control. The pipette resistances were monitored by application of 5mV pulses and were 10-20MΩ in the external solution. High resistance seals (>1GΩ) were made onto cell bodies or processes, and the membrane beneath the patch pipette
tip disrupted either by further suction or by application of a large, brief voltage pulse ("zap"). Entry into whole-cell mode was monitored using small voltage steps to evoke capacity transients. The cell was kept in whole-cell mode for 2 mins to allow the dyes to diffuse into the cell before removing the pipette. Done carefully, the membrane sealed over, leaving the cell body intact. Dye-filled cells were observed immediately after removing the patch pipette, using a mercury arc lamp and an FITC filter set. Once several profiles (3-8) were filled retinae were left on the stage of the microscope for 30 mins to allow time for the Neurobiotin to diffuse before fixing the tissue in 4% PFA in PBS. To aid recovery of the cell’s architecture on the confocal microscope, sketches were made of the position of labelled cells relative to the boundary of the retina.

2.8.2 Neurobiotin histochemistry

The tissue was processed to render the Neurobiotin visible using a protocol adapted from Becker and Davies (1995). Whole retinae were fixed overnight in 4% PFA in PBS at 4°C. Tissue was then washed 3x10 mins in PBS and blocked/permeabilised for 4-6h at RT in 0.1% Triton X-100 (Sigma), 0.1M L-lysine (Sigma) in PBS. Lysine reduces the non-specific binding of the primary antibody in chick retinal preparations (D.L. Becker, personal communication). Tissue was washed again (3x10 mins in PBS) prior to being incubated overnight at 4°C in Cy3-streptavidin (Molecular Probes) diluted 1:100 in PBS. Samples were then washed 4x30 mins in PBS before mounting them in Citifluor (glycerol/PBS) under thin glass cover slips. Cover slips were sealed to the slide using nail varnish (Boots, UK).

2.8.3 Confocal imaging of filled cells

Flat-mounted retinae were viewed on a confocal microscope (Zeiss LSM 510). Fluorescent profiles were located using epifluorescence illumination and images were acquired using Zeiss LSM510 software. xy-sections, approximately 1-2 μm apart, were taken throughout the depth of the retina, averaging each line 4 times. The fluorescence of FITC and of Cy-3 was sequentially excited using the 488nm line of argon laser and the 543nm line of the HeNe laser, respectively. Separation of the fluorescence signals of these two dyes is almost complete at these wavelengths. Three-dimensional projections of the full xyz stack were made and the projection was then rotated through
90° about the x-axis to produce an xz view and the final image produced using PaintShopPro 5.0 and 6.0 and Adobe Photoshop 5.0 software packages.

2.9 Immunocytochemistry

2.9.1 ChAT staining

Both sections and whole mounts were used for immunocytochemical labelling. For sections, E6 retinae were fixed in 4% PFA/PBS for 2h before being transferred to 20% sucrose/PBS overnight at 4°C. The eyes were embedded in O.T.C. and frozen. Transverse sections 20μm thick were taken using a cryostat and affixed to poly-L-lysine coated slides. Sections passing through the centre of the retina, adjacent to the optic nerve were processed. A blocking solution (0.1% Triton-X-100, 0.1M l-lysine in PBS), containing a monoclonal goat-anti ChAT primary antibody (1:200; Cambridge Biotech, UK), was applied for 4h at RT. After rinsing with PBS, the sections were incubated with an anti-goat biotinylated secondary antibody for 4h at RT (1:150; Vector, UK). Following a further 3x10min rinse with PBS, Cy-3-tagged streptavidin (1:150; Vector) was applied overnight at 4°C. Sections were rinsed in PBS (3x10mins), mounted in citifluor and held in place with a cover slip glued on with nail varnish before being imaged on a confocal microscope. The fluorescence of Cy-3 was excited using the 543nm line of a HeNe laser. Negative controls consisted of retinae processed as above but in the absence of primary antibody.

2.9.2 TuJ-1 staining

TuJ-1 staining for whole mounts

Retinae from E5 or E6 chicks were dissected out, flattened and fixed by immersion in 4% PFA/PBS overnight at 4°C. After 3x30min rinses in PBS the retina were permeabilized with 1% Triton X-100 in 0.1M L-lysine and PBS (blocking solution) for 4h (RT), and then incubated overnight at 4°C in blocking solution containing a monoclonal mouse-anti TuJ-1 primary antibody (1:500; Cambridge Biotech). After rinsing in PBS, retinae were incubated in anti-mouse Cy-5 tagged secondary antibody (1:200; Cambridge Biotech) for 6h at RT on a rocking plate. Retinae were washed in
PBS containing 2µM Hoechst and mounted in Citifluor beneath a glass cover slip before being imaged on a confocal microscope. The fluorescence of Hoechst and of Cy-5 was sequentially excited using the 350nm line of the UV laser and the 650nm line of the HeNe laser, respectively. Negative controls consisted of retinae processed as above but in the absence of primary antibody.

**TuJ-1 staining for co-localisation with NT response**

For experiments involving the identification of differentiated neurons within retinae used in Ca\(^{2+}\) imaging experiments the following protocol was used. Retinae from E6 chicks were loaded with Fluo-4 and Hoechst, as described in 2.2.1, and changes in chromatin and \([\text{Ca}^{2+}]\), imaged during drug application. As the chick retina lacks conspicuous landmarks, processing of the retina on the stage of the microscope without moving the preparation was found to be absolutely necessary in order to be able to find the region visualized during Ca\(^{2+}\) imaging. The retinae were fixed by immersion in 4% PFA/PBS for 2h. Following 3x30mins rinses in PBS; the tissue was permeabilized for 12h using 1% Triton X-100 in 0.1M L-lysine and PBS (blocking solution). Blocking solution containing a monoclonal mouse-anti TuJ-1 primary antibody (1:500; Cambridge Biotech) was then applied for 18h. After rinsing in PBS, retinae were incubated in anti-mouse Cy-5 tagged secondary antibody (1:200; Cambridge Biotech) for 24h. After rinsing off the excess secondary antibody with PBS containing 2µM Hoechst, the retinae were re-imaged on the confocal microscope (LSM510, Zeiss). The same region and confocal plane used for Ca\(^{2+}\) imaging was identified using the Hoechst staining as a guide and the distribution of the Cy-5-tagged TuJ-1 antibody was imaged in this plane and in x3 sections (1µm steps) immediately above and below this plane. Negative controls consisted of retinae processed as above but in the absence of primary antibody. The procedures were all conducted at RT.

The requirement for the retina to be left in place on the stage of the microscope made this experiment very difficult to complete. Having the VZ towards the microscope lens (necessary for the acquisition of good Ca\(^{2+}\) signals) required that this region was also immediately adjacent to the glass bottom of the perfusion chamber and permitted only limited access to antibodies. This necessitated very long application times, and tied up
the confocal microscope for up to 4 days at a time. The difficulty of this experiment meant it was only performed satisfactorily once, for the application of GABA.

2.9.3. Connexin staining

20µm frozen retinal sections from E5 chicks were permeabilized with 0.5% Triton X-100 in 0.1M L-lysine and PBS (blocking solution) for 4h (RT), and then incubated overnight at 4°C in blocking solution containing primary anti-Cx43 antibody (1:100). Specific antipeptide antibodies were raised to sequences conserved between species and specific for Cx 43: Gap15 (rabbit polyclonal), and 1A (mouse monoclonal) (Becker et al., 1995). The tissue was then washed 3x15mins in PBS and incubated in Alexa 488-tagged secondary antibodies (1:200; Cambridge Biotech) for 1h in the dark at RT. Retinae were washed 3x15mins in PBS. The final wash contained 2µM Hoechst. Retinae were mounted in Citifluor beneath a glass cover slip before being imaged on a confocal microscope. The fluorescence of Alexa 488 was excited using the 488nm line of an argon laser. Negative controls consisted of retinae processed as above but in the absence of primary antibody or with the primary antibody absorbed in excess of the peptide against which it was raised.
Figure 2.1. The perfusion system. Normal or drug-containing Krebs' solution was drawn from a reservoir by a peristaltic pump. This fed a constant level chamber suspended at a level higher than that of the microscope so that solution flowed into the perfusion chamber under gravity alone. The temperature of the perfusion chamber and of the solution feeding it was heated by a thermostatically controlled element and the temperature raised to 36°C. The rate of flow into the perfusion chamber was controlled by means of a clamp. Solution was removed from the chamber by a second peristaltic pump feeding into a waste container.
Figure 2.2. Ratiometric measurements of spontaneous $[Ca^{2+}]_i$ transients in dividing and migrating PCs. A, Dual-emission xy confocal image of the retinal VZ in a Fluo-4 (green) and Indo-1 (blue) loaded E5 retina. Three time points showing a spontaneous transient in a single cell (outlined). B, Changes in fluorescence intensity of Fluo-4 (green line) and Indo-1 (blue line) taken from the region shown in A. Fluorescent intensities were measured every 4s. Upward deflections in Fluo-4 and downward deflections in Indo-1, respectively, represent an increase in $[Ca^{2+}]_i$. A.U. arbitrary units. C, Time-course of changes in Fluo-4/Indo-1 ratio signal. Upward deflections in ratio signals represent an increase in $[Ca^{2+}]_i$. D-G, The same measurements made for a migrating cell. Images are ‘virtual z-scans’ (see text) through a fold in a retina and a single migrating cell has been highlighted.
Figure 2.3. Measurement of the time spent in metaphase during mitosis and rotations of the mitotic apparatus. Confocal images of VZ cells labelled with Hoechst 33342. Images were acquired at 15s intervals but examples have been extracted from the movie to show key stages. Interphase (0mins) is followed by prophase (5mins) during which the cell’s chromatin condenses. The transition between prophase and metaphase was rapid (5.5mins). During metaphase the mitotic apparatus rotates within the plane of the VZ. Upon entering anaphase (29mins) the pairs of chromosomes pull apart at a constant angle (31-33mins). The orientation of the metaphase rod at time point $n$ was measured relative to the final angle adopted by the chromatin of that cell at anaphase ($\alpha$, see 5.5mins). A line was drawn along the longitudinal axis of the metaphase rod (red lines). The orientation at anaphase was determined by drawing a line longitudinally through the two parting clusters of chromatin (blue lines). The mean angle at anaphase (green lines) is shown on each of the images. Scale bar: 5µm.
Figure 2.4. Measurement of eye diameter and the angle of division. A, Low power CCD image of a whole embryo. The eye diameter (δ) was measured through the choroid fissure, across the widest part of the eye. This was repeated for both eyes. B, Confocal image of a retinal section labelled with Hoechst 33342. The angle of division was measured for those profiles clearly in metaphase-telophase, where the highly condensed chromatin has a rod-like appearance (e.g. open arrow). Line 1 is parallel to the plane of the RPE. Line 2 is a line perpendicular to the RPE and passes through the centre of the metaphase rod. Line 3 is a line passing through, and at the same orientation as, the rod. The acute angle between lines 2 and 3 was measured and defined as α. An angle of 0° corresponds to a division in which the metaphase rod is aligned at right angles to the RPE (a vertical division) whilst a division in which the metaphase rod lies parallel to the RPE has an angle of 90° (horizontal division). Inset, the angle of division of cells in anaphase was measured by drawing a line through the two separating clusters of chromatin and measuring the angle between this and a line at right angles to the RPE.
Figure 2.5. “DiOlistic” labelling of the retina. Tungsten particles coated with lipophilic dyes were shot into neural retinal tissue using a high pressure helium 'gene gun'. In order to prevent clusters of dye-coated particles from landing on the preparation, track-etched membrane filters were interposed between the gene gun and the target tissue. Density of labelling was controlled by altering the gas pressure or by varying the distance between the gun and the preparation (5–15 mm).
Figure 2.6. Reconstruction of 3D time-series. A, A single 3D stack is created by taking a series of xy scans at 1μm steps throughout the depth of the retina at t₀ and repeated at 15min intervals (t₁, t₂ etc). B, Each series of xy scans was built into a 3D xyz block and projected through the xz plane to form a 2D image. C, This was repeated for each time point to make a 2D movie of the block. The distance (δ) moved by individual progenitor cells (shown in red) at each time point was then measured.
Figure 2.7. Measurement of interkinetic nuclear migration. A, The centre of each cell body was taken as the centre of a box drawn around the bulge containing the nucleus. The distance between the VZ (vertical line) and the centre of the nucleus was measured at each time point (A-D). C, D, Process length was measured from the centre of the nucleus to its tip (arrow head).
Chapter 3

Confocal imaging of spontaneous and neurotransmitter-evoked $[Ca^{2+}]_i$ signals in the VZ of the developing chick retina

3.1 Introduction

The production of cells by CNS PCs, and the determination of their fate, is regulated by an array of intrinsic and extrinsic signals. Many in vitro studies have implicated a developmental role for both slow and fast neurotransmitters, prior to the onset of synaptogenesis. Purinergic (Sugioka et al., 1996), muscarinic (Sakaki et al., 1996), GABAergic (Yamashita and Fukuda, 1993a) and glutamatergic (Sugioka et al., 1998) stimulation of the embryonic chick retina during neurogenesis have all been shown to cause increases in $[Ca^{2+}]_i$. Intracellular $Ca^{2+}$ is a key influence on developmental events in the CNS and has been implicated in the regulation of differentiation, migration, cell fate and circuit formation. $[Ca^{2+}]_i$ transients are associated with progression through checkpoints in the cell cycle (Ciapa et al., 1994; see Santella, 1998 and Santella et al., 1998 for reviews) and are correlated with events such as nuclear migration, nuclear envelope breakdown, the transition from metaphase to anaphase, and cytokinesis. In the VZ of the neocortex, individual cells display intermittent spontaneous $[Ca^{2+}]_i$ transients (Owens & Kriegstein, 1998). These transients do not depend on the activation of voltage-sensitive $Na^+$ channels, VGCCs or amino acid neurotransmitter receptors. Owens and Kriegstein (1998) have put forward the hypothesis that $[Ca^{2+}]_i$ activity can act to influence the cell cycle in the embryonic CNS.

Our knowledge of the responsiveness of the embryonic retina to neurotransmitters is based largely on immunocytochemical and $Ca^{2+}$-imaging techniques (Yamashita and Fukuda, 1993a; Yamashita et al., 1994; Sakaki et al., 1996). However, immunocytochemical techniques give no indication of whether the receptors labelled are functional or what their possible actions may be. $Ca^{2+}$-imaging detects receptors by virtue of the resultant influx of $Ca^{2+}$ through the receptor itself or by release from intracellular stores. Most of the techniques applied thus far have not employed confocal microscopy and so do not permit identification of the cells that respond. Here, confocal
microscopy and labelling of cells with a combination of the vital chromatin dye Hoechst 33342 and the Ca\(^{2+}\) indicator Fluo-4 have been used to relate spontaneous changes in [Ca\(^{2+}\)]\(_i\), as well as those evoked by muscarinic, purinergic, glutamatergic and GABAergic stimulation, to the mitotic status of the cells in the VZ of the embryonic chick retina. The basic pharmacology and nature of [Ca\(^{2+}\)]\(_i\) changes in response to each of these neurotransmitters is described, along with the ability of cells in the VZ to respond to more than one of them, and how the pattern of these responses changes during early development.

Neurotransmitters play important roles in the refinement of developing neural circuits (e.g. Wong, 1999; Sernagor et al., 2001). However, there is increasing evidence to suggest that they may also play a role at earlier stages in development (LoTurco et al., 1995). Establishing the identity of the cells that respond to different neurotransmitters is important for elucidating the potential actions that neurotransmitters may have in development. For example, are actively proliferating PCs capable of responding to such signals or do neurotransmitters only act upon those cells that have undergone terminal division? Given that the expression of both neurotransmitters and their receptors changes with time (see Introduction) it is important to establish how these changes relate the rapid changes in proliferation and differentiation that occur during the first developmental week. The experiments presented in this chapter attempt to address these issues.
3.2 Methods

The methods used in this chapter are described in detail in Chapter 2 (section 2.4). Briefly, retinae were dissected and loaded with Fluo-4 AM for 1h at RT (for exogenous agonist applications) or at 36°C (for spontaneous activity). Ten minutes prior to the end of Fluo-4 loading, the vital chromatin dye Hoechst 33342 was added to the loading medium. After loading, the retinae were maintained in gassed Krebs’ solution at RT or 36°C (as above). Retinae were transferred to the stage of a microscope and superfused with Krebs' solution and imaged at either RT or at 36°C (as above). When imaging spontaneous [Ca^{2+}]_i activity, pairs of retinae from one embryo were used wherever possible; one was used as a control and the other to examine the effects of drug application. The order of the use of control vs drug retinae was alternated between experiments.

Retinae were either imaged as flat mounts, with the VZ facing the objective, (for spontaneous and evoked activity in the VZ) or as ‘virtual z-sections’ (for spontaneous activity through the depth of the retina), as described in section 2.4.2. Unless otherwise stated, images were taken at 4s intervals and analyzed off-line. The mean fluorescence of individual cells was normalized to the initial value at time 0 (ΔF/F). Increases in the fluorescence of Fluo-4 reflect increases in [Ca^{2+}]_i. A change in fluorescence in excess of a criterion level of 10% above baseline was taken as a response (see section 2.4.2). Figures show single confocal sections through the VZ. Unless otherwise stated, traces show the mean change in fluorescence from a region of interest (ROI) selected to represent [Ca^{2+}]_i changes within the soma of single cells. Dual labelling with Hoechst 33342 to stain DNA enabled identification of the cells mitotic status.

All data presented in this chapter were normally distributed, as determined using the Kolgomorov-Smirnov test (GraphPad Prism 3.00). Therefore, data was tested using an unpaired Student’s t-test. Differences were considered statistically significant at one of two levels: *P<0.05 and **P<0.01. Results are given as means ± SEM of each control or test group, where N=number of retinae investigated and n=number of cells recorded.
3.3 Results

3.3.1 Simultaneous recording of [Ca\(^{2+}\)]\(_i\) signals and mitotic status by confocal microscopy

Whilst several previous investigations have examined neurotransmitter-induced responses in the developing retina, the majority have used non-confocal imaging techniques. It has, therefore, not been possible to accurately identify which cells respond to a given manipulation. For example, responses may arise from actively dividing PCs, PCs at other stages in the cell cycle, or from NDCs. Here, confocal microscopy has been employed to investigate whether or not cells in the embryonic retinal VZ, the only region in which division occurs, respond to neurotransmitters. Dual labelling of the retina with Fluo-4 and Hoechst 33342 allows simultaneous imaging of [Ca\(^{2+}\)]\(_i\) and chromatin. The VZ consists of a single layer of cells thus individual xy confocal scans allow the simultaneous imaging of a large number of VZ cells.

3.3.2 Interphase and mitotic cell populations in the VZ

Incubation of E6 chick retinae in Fluo-4 AM leads to labelling of the majority of the cells in the VZ. Two populations can be identified on the basis of cell diameter and the intensity of Fluo-4 labelling. One consists of large dark profiles (4.6\(\mu\)m±0.2 diameter, \(N=4, n=80\)) and the other of smaller (2.7\(\mu\)m±0.2, \(N=4, n=80\)), more brightly labelled cells. Figure 3.1a shows an example of Fluo-4 loading in an E6 retina. Comparison of Fluo-4 labelling with that of the same retina by Hoechst 33342 shows that the majority of the large dark cells contain mitotic figures, while the chromatin of the more brightly labelled somata is typical of that of interphase nuclei (Figure 3.1b). It is possible that the brightly labelled cells fluoresce more strongly because they are unhealthy or damaged. Alternatively, the dimly labelled cells may be poorly loaded with the fluorescent indicator. However, when Krebs' solution containing high [K\(^+\)] (20mM)\(^1\) was used to depolarise cells the majority of both interphase and mitotic cells showed large increases in fluorescence (data not shown). This suggests that both populations were sufficiently

\(^1\) Solution contained (in mM): 86 NaCl, 30 NaHCO\(_3\), 20 KCl, 1 MgCl\(_2\), 3 NaH\(_2\)PO\(_4\), 1 CaCl\(_2\) and 20 Glucose (pH 7.4 by gassing with 5% CO\(_2/95\%\) O\(_2\)).
well loaded with Ca\(^{2+}\) indicator to be able to respond to the changes in [Ca\(^{2+}\)], brought about by moderate depolarisation with raised [K\(^+\)], and that few cells were damaged during the dissection and loading procedures.

Labelling with TuJ-1 (Figure 3.1c), an antibody for neuron-specific β-tubulin and a marker of post-mitotic neurons (Moody et al., 1989; Lee et al., 1990), shows that about a third of the VZ’s interphase cells (36±3%, N=3, n=212) are TuJ-1\(^+\) at E6, while less than 2±1% (N=3, n=85) of cells containing mitotic figures stain for TuJ-1. In contrast, imaging the immature GCL at the same age shows widespread labelling of cell bodies and the developing fibre layer (Figure 3.1d). Thus, at E6 the VZ layer consists of mitotic cells, which are large, label dimly with Fluo-4 AM and are seldom TuJ-1\(^+\), and a brightly labelling interphase population that contains a mixture of PCs (TuJ-1\(^-\) and making up about two-thirds of the total interphase population) and differentiating neurons (TuJ-1\(^+\)). Immunocytochemical labelling of glial cells or PCs was not performed in these investigations. However, Müller cells are likely to be present only in small numbers at E6 since the majority are thought to be born after neurogenesis is complete, at around E9 in the chick (Prada et al., 1991; Linser et al., 1997).

3.3.3 Spontaneous [Ca\(^{2+}\)]\(_i\) fluctuations in the VZ

To examine the possibility that retinal VZ cells produce spontaneous [Ca\(^{2+}\)]\(_i\) fluctuations, E6 retinae were imaged in the xy plane whilst being superfused with Krebs’ solution at 36°C. Many cells exhibited spontaneous and intermittent [Ca\(^{2+}\)]\(_i\) transients (Figure 3.2). The majority of these events did not propagate into neighbouring cells (but see Chapter 6). Examples of spontaneous events in typical retinae are shown in Figure 3.2a. These transients occurred at low and roughly equal frequency (~1 event per cell every 5mins) in both mitotic and interphase cells with a tendency for the latter group to be more active (see below). Such spontaneous [Ca\(^{2+}\)]\(_i\) activity was rare at RT. The sampling rate of 0.25Hz (one image every 4s) used in these experiments meant that the fastest event that could be resolved was of ~8s duration. To ensure that faster events were not being missed, several experiments were performed in which images were acquired at the faster sampling rate of 1Hz. Figure 3.2b shows a transient from a cell using a frame rate of 1Hz; this event lasted 15s with a duration at
half-peak of 5s. The events from a sample of 64 cells at E6 had a mean duration at half-peak of 13.8±1.3s (range, 4-57s), and the fastest event lasted ~4s. These results indicate that the [Ca^{2+}]_{i} transients in VZ cells have a relatively slow onset and last for several seconds and the majority are likely to be detected using the sampling frequencies employed here.

Simultaneous spontaneous [Ca^{2+}]_{i} activity also occurred in neighbouring cells. These co-ordinated events sometimes appeared to represent the spread of transients between cells prior to cytokinesis (Figure 3.2c) but were also seen between adjacent interphase cells. More extensive wave-like spread of the transients was also observed, in which the wave invaded large numbers of cells (Figure 3.2d). These wave-like events are discussed in more detail in Chapter 6.

3.3.4 Cells throughout the depth of the retina undergo spontaneous [Ca^{2+}]_{i} transients

PCs and NDCs typically have a bipolar morphology with processes extending to the VZ and the GCL. The ventricular processes terminate amongst the cell bodies of cells in the VZ. In retinae loaded with Fluo-4 and imaged in the xy plane, these processes appear as small profiles <1μm diameter, surrounding cell bodies on the VZ (arrow heads, Figure 3.2a). These small processes were observed to undergo transient changes in fluorescence suggesting that cells deeper in the retina may undergo [Ca^{2+}]_{i} transients similar to those observed in the VZ and that these may propagate along the length of the cell.

Confocal imaging of the xz plane of the retina in ‘virtual z sections’ showed that spontaneous [Ca^{2+}]_{i} transients were observed in cells throughout the depth of the neural retina at a frequency similar to that seen in the VZ (~1 event/5mins). The mean duration at half-peak of a random sample of 50 cells was 26s (range, 3-70s). However, two types of event were seen; 37/50 cells displayed short duration transients (8±0.7s), while the remaining cells exhibited much longer lasting transients with half-peak duration of 43±8s. Figure 3.3 shows a ‘virtual z-section’ in which 5 cells have been outlined. The changes in [Ca^{2+}]_{i} in these cells are shown in the traces below. During a 500s imaging period, each of the cells shown displayed single [Ca^{2+}]_{i} transients.
In the experiments described above, the point of origin of the \([Ca^{2+}]_i\) transients was not determined. In 4 preparations the scan-time was reduced to the fastest possible (0.98s) and the speed of propagation determined as the difference in time taken for \([Ca^{2+}]_i\) to increase by 10% in ROI along a PC’s length. The speed of propagation was variable but the majority spread the length of the cell in the time between two scans. Figure 3.4a shows a cell in which a rise in \([Ca^{2+}]_i\) occurs apparently simultaneously throughout the cell. The speed of propagation of these fast events must be greater than 45\,\mu m/s (n=12) since the transient propagates 44\,\mu m within the 0.98s between the acquisition of two images. Other transients propagate more slowly. An example of these slower transients is shown in Figure 3.4b. A rise in \([Ca^{2+}]_i\) occurred in the cell’s VZ process, then spread towards the cell body and then on into the basal process; the whole event lasted ~40s. The rate of propagation from the end of the VZ process to region 5, which lies on the further edge of the nucleus, was ~10\,\mu m/s. The time to peak of the \([Ca^{2+}]_i\) transient was rapid in the VZ process (t_{50} = 8s) but was much slower in the nucleus (t_{50} = 19s). In other cells transients propagated in the opposite direction, moving along the vitreal process towards the ventricular region of the cell (data not shown).

3.3.5 The proportion of VZ cells showing spontaneous \([Ca^{2+}]_i\) transients decreases between E4 and E6

In order to determine whether there were any differences in the pattern and frequency of spontaneous \([Ca^{2+}]_i\) activity at E4 and E6, retinae were imaged for periods of 500s at a sampling frequency of 0.25Hz. At E4 spontaneous events were seen in 48±3% of cells (N=4, n=239) during a 500s period. The proportion of interphase cells undergoing spontaneous activity was almost twice that of the mitotic population with 57±0.5% (n=120) and 32±6% (n=119) of cells active, respectively. At E6, the proportion of cells showing spontaneous \([Ca^{2+}]_i\) events was lower than at E4 (27±2%, N=5, n=372, P<0.01) and the transients occurred with equal frequency in the mitotic and interphase populations (26±2%, n=172 of mitotic cells and 28±2%, n=200 of interphase cells were spontaneously active). The mean duration of the transients at E4 and E6 was 11.7±1secs (n=50) and 13.8±1.3 secs (n=64) respectively; these values are not statistically significantly different.
3.3.6 *Spontaneous \([Ca^{2+}]_i\) activity in the VZ is independent of Na\(^+\)-dependent action potentials*

Neurotransmitters are present in the retina from early times in development (see section 1.6). Therefore, experiments were carried out to examine whether action potential-dependent neurotransmitter release mediates the spontaneous \([Ca^{2+}]_i\) transients observed in the VZ. Spontaneous activity was measured during and after the application of the Na\(^+\)-channel blocker TTX (10\(\mu\)M). TTX failed to reduce the frequency of spontaneous activity, as is shown in Figure 3.5. 46±2\% of cells showed spontaneous activity in a 500s period in the presence of TTX (N=3, n=122) compared to 39±11\% (N=3, n=122; P=0.52) after wash with control solution. Thus, the occurrence of spontaneous \([Ca^{2+}]_i\) transients appears to be independent of the production of Na\(^+\)-dependent action potentials.

3.3.7 *Spontaneous \([Ca^{2+}]_i\) activity in the VZ results from endogenous release of neurotransmitter*

Purinergic, muscarinic, GABAergic and glutamatergic stimulation of the developing chick retina have all been shown to cause increases in \([Ca^{2+}]_i\), though the cells responding have not always been identified. The experiments described below tested the possibility that the spontaneous \([Ca^{2+}]_i\) transients observed in the VZ (section 3.3.3) originated from endogenous activation of one or more neurotransmitter receptors within the E6 retina. The frequency of the spontaneous events in retinae exposed to the antagonists pirenzpine (to block mACh receptors), suramin (to block P2 receptors), NBQX (to block AMPA receptors) or bicuculline (to block GABA\(_A\) receptors) (all 25\(\mu\)M) was measured and compared with those in controls. The frequency of \([Ca^{2+}]_i\) transients was lower in the presence of each of the four antagonists than in controls (Figure 3.6). Pirenzpine reduced the production of transients in both mitotic and interphase cells, by 75±7\% (N=5, n=500, P<0.03) and 68±9\% (N=5, n=500, P<0.02) respectively, compared with controls. Similarly, application of suramin (investigated at E4, see below) reduced activity in mitotic cells by 55±15\% (N=5, n=250, P<0.02) and by 69±12\% in the interphase population (N=5, n=250, P<0.03). The effects of two other purinergic antagonists, PPADS and Reactive Blue were investigated for the purposes of further experiments discussed in Chapter 4. PPADS (30\(\mu\)M) reduced spontaneous...
activity in mitotic and interphase cells by 58±2% (N=3, n=100, P<0.03) and 64±2% (N=3, n=120, P<0.01), respectively, compared to E4 controls. Reactive Blue (25 μM) reduced the activity in mitotic cells by 75±2% (N=3, n=89, P<0.01) and by 70±4% (N=3, n=120, P<0.04) in the interphase population. NBQX had a similar, but smaller, effect reducing [Ca^{2+}]_i transient production in the mitotic population by 38±9% (N=6, n=407, P=0.059). The effect on the interphase population was more marked with activity reduced by 58±9% (N=6, n=613, P<0.02). Bicuculline had little effect on [Ca^{2+}]_i transients in the mitotic population (N=6, n=182, P=0.56) but reduced their occurrence in interphase cells by 68±10% (N=6, n=289, P<0.02). Application of all four antagonists simultaneously reduced activity in the combined interphase and mitotic population by 89±6% (N=3, n=150, P<0.01). See Table 1.

3.3.8 Spontaneous [Ca^{2+}]_i activity in the VZ is enhanced by eserine and iso-OMPA and reduced by apyrase

That spontaneous [Ca^{2+}]_i activity in the VZ can be reduced by inhibition of muscarinic and purinergic receptors would suggest this activity arises from the endogenous release of neurotransmitters. The level of spontaneous activity would, therefore, be expected to increase if the endogenous levels of ACh or ATP were raised, and conversely decrease if the levels were reduced. To investigate this possibility pharmacological tools were used to manipulate endogenous [ATP] or [ACh] levels.

The presence within the retina of choline-acetyl transferase (ChAT), an enzyme involved in the formation of ACh from choline and acetyl Co-A, supports the idea that endogenous release of ACh may be involved in the initiation of spontaneous [Ca^{2+}]_i transients. Immunocytochemistry using antibodies directed at ChAT leads to labelling in the chick retina at early times (E4-6). ChAT labels cells throughout the retina including the VZ (Figure 3.7a-d).

The levels of [ACh]_o can be increased by preventing its breakdown. The enzyme AChE, which is inhibited by the drug eserine, cleaves, and thus inactivates, ACh. In addition, the early embryonic avian retina also contains a significant level of BuChE (Layer et al., 1987; for review, see Layer 1991), which is inhibited by iso-OMPA (tetraisopropyl
pyrophosphoramide). Both AChE and BuChE are able to cleave >10,000 molecules of ACh per second (Bazelyansky et al., 1986, see also Rang et al., 1999). E6 retinae were split in two and one piece was perfused with solution containing eserine and iso-OMPA (both 100μM), and the other with normal Kreb’s. As shown in Figure 3.8a, the number of cells showing spontaneous [Ca^{2+}]_i activity increased by 255% (P<0.01) with 65±5% of cells (N=7, n=345) active in the presence of iso-OMPA/eserine, compared with 25±2% in controls (N=7, n=372). These drugs also increased the frequency of events seen in individual cells. In cells showing spontaneous activity, the average number of transients in a 500s period was 1.3±0.1 event/cell (N=5, n=69) in controls. This value increased to 2.3±0.1 events/cell (N=5, n=163, P<0.01) in the presence of Iso-OMPA/eserine. The distributions of the frequency of spontaneous activity in the presence of iso-OMPA/eserine compared with controls are shown in Figure 3.8b.

ATP released into the extracellular space is usually degraded by ATPases. The endogenous levels of ATP can be artificially lowered by application of ATP diphosphohydrolase, commonly called apyrase. Both apyrase and ATPase hydrolyse ATP to ADP+P_i and AMP+2P_j. Apyrases have low substrate selectivity and catalyse the hydrolysis of di- and tri-phosphate nucleotides. There is an age-dependent change in the [Ca^{2+}]_i response evoked by purine nucleotides (see section 3.3.18). Purine nucleotides evoke responses in large numbers of ventricular cells at E4, but only a small number of cells respond in the E6 retina. Therefore, E4 retinae were used to investigate the effects of apyrase. E4 retinae were split into two pieces and one half was incubated for 20mins in, and then perfused with, normal Krebs’. The other was first incubated for 20mins in, and then perfused with, Krebs’ solution containing apyrase (80U/ml; 1 unit of apyrase liberates 1.0μmole of inorganic phosphate from ATP or ADP at pH6.5 at 30°C). In these experiments, which used expensive reagents, the perfusion rate for both control and apyrase-treated retinae was reduced to the minimum required to keep the temperature and pH stable. The number of cells showing spontaneous activity during the 500 sec recording interval fell from 29±2% (N=3, n=175) in controls to 8±3% (N=3, n=170; P<0.01) in the presence of apyrase, a 73±10% reduction (Figure 3.9)
3.3.9 ACh produces changes in \([\text{Ca}^{2+}]_i\) in VZ cells in the early chick retina

In order to determine the size and nature of the population of cells that respond to the neurotransmitters ACh, ATP, glutamate and GABA, specific agonists were applied to E6 retinae to stimulate purinergic, muscarinic, GABAergic and glutamatergic receptors.

The neurotransmitter ACh and its muscarinic receptor are thought to be present as early as E3.5 in the chick retina (Sugiyama et al., 1977; Sakaki et al., 1996). Confocal imaging shows that ACh (100μM) produces marked increases in \([\text{Ca}^{2+}]_i\) in a significant proportion of VZ cells between the ages of E4 and E6. Responses were seen in 71±10% (N=3, n=209) of cells at E6. The response of individual cells was usually oscillatory (Figure 3.10a). The extent of the oscillations was dependent upon concentration of the agonist. Exposure to 50μM ACh for 2mins evoked an average of 3.4±0.1 oscillations/cell (n=73, N=3)

3.3.10 Pharmacology of the cholinergic response

ACh receptors may be one of two types: nicotinic or muscarinic. Agonists and antagonists were used to determine the nature of the cholinergic response in E6 chick retina. The mAChR antagonist atropine (10μM) virtually abolished the rise in \([\text{Ca}^{2+}]_i\) produced by 50μM ACh (1±1%, N=3, n=150, see Figure 3.13). The effects were partially reversible although the responses following washout were generally smaller than those to the initial application.

Most cells (94±2%, N=10, n=671) responded to the mAChR agonist CCh (50μM). These responses were similar to those produced by ACh and were also usually oscillatory (Figure 3.10b). The number of cells responding to CCh increased in a dose-dependent manner (Figure 3.10c). Applications of 0.5μM, 1μM, 10μM, 50μM and 100μM CCh were applied to separate pieces of retina to avoid desensitisation. Small numbers of responses were evoked by 0.5μM of the drug while ~50% of cells responded when retinae were challenged with 10μM CCh (see figure legend for details).
Nicotine (100μM), produced responses in less than 6±2% (N=6, n=407) of cells, while muscarine (100μM) evoked [Ca^{2+}]_i transients in 87±10% (N=4, n=265) (Figure 3.11), showing that the muscarinic agonist CCh, which acts with low potency at nicotinic receptors (A. Gibb, personal communication), produced its effects in most cells in the VZ via activation of mAChRs. Since CCh produces responses similar to that of ACh, and is more stable, CCh was chosen as the muscarinic agonist for the experiments described below. See Table 2.

Atropine is a broad-spectrum mAChR antagonist. In order to determine the subtype of mAChR involved, the effects of specific antagonists of mAChRs were investigated. The application of pirenzipine (25μM), the M_1^2 subtype-specific antagonist reduced the number of cells responding to CCh (50μM) from 94±2% (N=4, n=671,) to 9±4% (N=4, n=340; P<0.01) (Figures 3.11b and 3.12). This was surprising given that the chick retina is thought to express only cm2, cm3 and cm4 (Fischer et al., 1998a). However, the chick M_2 and M_4 receptors have an anomalously high affinity for pirenzipine (Tietje et al., 1991). Therefore, the M_2 specific antagonist AFDX 116 (up to 1mM) was applied. Whilst the response to 50μM CCh was markedly reduced by AFDX 116, (by 55±9%; n=198, N=7; P<0.01), it was notably less effective than pirenzipine, with almost half the cells still able to respond to CCh. The cloned chick cm4 receptor demonstrates a moderate affinity for AFDX 116 (Tietje et al., 1990). The M_4-selective antagonist tropicamide (25μM) virtually abolished the response to CCh (100μM) in the E6 chick retina. Only 2±1% (n=270, N=4) of cells responded to CCh in the presence of the antagonist, compared to 93±3% (n=310, N=4; P<0.01) in controls. The block was fully reversible; 99±0.1% (n=190, N=3) cells responded following the removal of the antagonist from the external solution. The pharmacology of the response to cholinergic stimulation in cells of the early embryonic chick retinal VZ is consistent with that of the M_4 muscarinic receptor although other mAChR subtypes may also be present. See Table 2.

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2 The chick muscarinic receptors are defined according to whether they have been classified by molecular (cm2, cm3 etc) or pharmacological (M_2, M_3 etc) means.
3.3.11 UTP produces changes in \([\text{Ca}^{2+}]_i\) in VZ cells in the early chick retina

UTP (50μM) produced marked increases in \([\text{Ca}^{2+}]_i\) in 11±5% (N=6, n=210) of VZ cells (Figure 3.14a) at E6. Since far more cells respond to purine nucleotides at E4 than at E6 (see section 3.3.18), E4 retinae were used to investigate the pharmacology of the purinergic response. The cellular response to UTP was usually oscillatory, although some cells exhibited a long-lasting plateau. The extent of the oscillations was dependent upon the concentration of the agonist. Exposure to 50μM UTP for 2mins evoked an average of 2.4±0.1 oscillations/cell (n=229, N=4). Figure 3.14b shows the dose-response relationship for UTP for E4 chick retina. Increasing concentrations caused a rise in the number of cells showing changes in fluorescence in excess of the 10% ΔF/F criterion level. Significant responses were seen to concentrations as low as 250nM and near maximal numbers of cells responded upon application of 10μM UTP.

3.3.12 Pharmacology of the purinergic response

Purinergic receptors are either ionotrophic (P2X) or metabotropic (P2Y). Despite the lack of good, commercially available agonists and antagonists selective for P2 receptors, an attempt was made to pharmacologically characterise the receptor involved in the purinergic response seen in VZ cells. Whilst the involvement of A1 and A2 (formerly P1) adenosine receptors was not investigated here, their involvement in the responses observed is highly unlikely. A1 and A2 receptors are thought to be expressed from ~E10 and E14, respectively (Paes de Carvalho et al., 1992). Sugioka et al., (1996) have shown that the early embryonic chick retina shows no change in \([\text{Ca}^{2+}]_i\) in response to adenosine. ATP (50μM) evoked responses in ~25% of E4 VZ cells (see below), a response that was blocked by the broad-spectrum purinergic antagonist suramin. The P2 agonist, UTP (50μM), evoked responses in ~75% of VZ cells. Suramin (25μM) blocked the response to UTP (N=6, n=384) with only 2±1% of cells showing a change in \([\text{Ca}^{2+}]_i\). The effects of suramin were reversible following a 15min washout (Figures 3.14a).

UTP shows reasonable selectivity for the P2Y receptor. Since responses could be obtained with low concentrations of UTP (see below), this suggests that a receptor of the P2Y class mediates the \([\text{Ca}^{2+}]_i\) responses to UTP observed in the early embryonic
chick retina. Six subtypes of the P2Y receptor have been described. Unfortunately, the accurate identification of these receptors in vivo is hindered by the lack of good, specific agonists and antagonists. However, there are some differences between the sensitivity of these subtypes to purine nucleotides. Below, the potency of four purine nucleotides at the purinergic receptor/s in the chick retina and the efficacy of some commercially available purinergic antagonists are established.

UTP (50μM) was three times more effective at stimulating cells in the VZ, than the presumed endogenous agonist, ATP (50μM). UTP evoked criterion responses in 72±7% (N=7, n=425) of cells, compared with the 24±4% (N=7, n=425; P<0.01) that responded to the same concentration of ATP. P2Y receptor subtypes show a differential sensitivity to tri- and di-nucleotides. Application of the uridine dinucleotide UDP (50μM) evoked criterion responses in fewer cells (9±2%; N=5, n=335; P<0.01) than either of the trinucleotides. However, the adenosine dinucleotide, ADP (50μM) evoked strong responses, producing criterion responses in 70±5% (N=4, n=250; P=0.98 compared to UTP) of VZ cells. Thus, the potency order for the purinergic response is UTP=ADP>ATP>UDP (Figure 3.15). Similar numbers of mitotic and interphase cells appeared to respond to each agonist, although this was not quantified. See Table 2.

Suramin is a non-selective purinergic antagonist, and has been reputed to have effects at receptors other than the P2 receptor (e.g. Cirillo et al., 2001), but it is relatively ineffective at the P2Y4 receptor. Similarly, whilst Reactive Blue acts at most of the P2Y receptors, the P2Y2 class is not inhibited. Co-application of 50μM Reactive Blue with 50μM UTP reduced the percentage of cells responding to the agonist from 70±9% to 8±3% (N=3, n=226, P<0.01) (Figure 3.16b). PPADS has been shown to block the P2Y1, P2Y4 and P2Y6 receptors in mammalian cells. The response to UTP was reduced from 78±3% to 6±1% of cells (N=3, n=160, P<0.01) in the presence of 30μM PPADS (Figure 3.16c). That Reactive Blue and PPADS block the response to UTP by an extent similar to that achieved with suramin strongly indicates that P2 receptors mediate the purinergic response in the chick retinal VZ. To achieve a full characterisation of the purinergic receptor subtypes involved in the response here would be very difficult since the efficacy of both agonists and antagonists is dependent upon both the species and tissue investigated. The pharmacology of the response to purinergic stimulation in cells
of the early embryonic chick retinal VZ is reasonably consistent with that of the cloned avian p2y3, although the involvement of other subtypes cannot be ruled out (for discussion see section 3.4). See Table 2.

3.3.13 GABA produces changes in $[Ca^{2+}]_i$ in VZ cells in the early chick retina

GABA is a major inhibitory neurotransmitter in the adult retina. However, it has a depolarising action in the immature nervous system (Segal and Barker, 1984; Cherubini et al., 1991). Application of GABA (100μM) evoked $[Ca^{2+}]_i$ transients in about a third of all VZ cells (35±8%; N=6, n=240) at E6. In contrast to the responses produced via the activation of metabotropic receptors by CCh or UTP, the increases in $[Ca^{2+}]_i$ evoked by GABA consisted of single transients that declined monotonically with time (Figure 3.17a).

The GABA-evoked responses were suppressed by the GABA$_A$ specific antagonist bicuculline (Figure 3.17a). The number of cells responding to 100μM GABA fell to only 4±4% (N=4, n=200) in the presence of 25μM bicuculline. The block was reversible and the GABA-evoked response returned once bicuculline was washed from the bath. These data support previous work (Yamashita and Fukuda, 1993a) indicating that GABA$_A$ receptors mediate the effects of GABA, the action of which is to depolarise retinal cells thereby activating VGCCs and permitting $Ca^{2+}$ entry. See Table 2.

3.3.14 Glutamate produces changes in $[Ca^{2+}]_i$ in VZ cells in the early chick retina

Biochemical studies suggest that glutamate receptors may not be present in the chick retina until E7 (Somahano et al., 1988). However, while elevations in $[Ca^{2+}]_i$ in response to NMDA do not appear until E8 (Allcorn, 1996), AMPA/kainate receptors have been shown to be present in E6 chick retina (Allcorn, 1996; Sugioka et al., 1998).

The action of glutamate on VZ cells was examined in E6 chick retinae. The responses produced by 100μM glutamate were similar in form to those produced by GABA and consisted of single, monotonically declining transients (Figure 3.17b). 39±11% (N=6, n=280) of cells in the VZ responded to glutamate. The AMPA/kainate receptor agonist AMPA (100μM) produced responses similar to glutamate with responses arising in
40±4% (N=3, n=282) of the VZ population (Figure 3.17c). The specific AMPA receptor antagonist NBQX (25μM) markedly reduced the response to both agonists (Figure 3.17b,c); only 6±2% (N=3, n=240, P<0.01) of VZ cells continued to respond to AMPA in the presence of NBQX whilst 10±5% (N=4, n=200) responded to glutamate in the presence NBQX. Whilst recovery of the glutamate response after washout of NBQX was seen in some preparations, others showed little or no response. Thus, to assess the inhibitory effects of NBQX, retinas were split in two. One piece was exposed to glutamate (control) and the other to NBQX+glutamate.

The NMDA receptor antagonist AP5 (25μM) had little effect on the response to glutamate (Figure 3.17c). 100μM glutamate produced responses in 38±4% (N=4, n=164) of cells in controls and in 34±4% (N=4, n=154; P=0.23) of cells exposed to AP5. These data provide further evidence that the response to glutamate at E6 is predominantly mediated via AMPA/kainate, rather than NMDA, receptors.

3.3.15 Muscarinic and purinergic responses involve the release of \([Ca^{2+}]_i\) from intracellular stores whilst GABAergic and glutamatergic responses result from entry of \(Ca^{2+}\) from the extracellular space.

The responses to CCh and UTP were unaffected by the \(Ca^{2+}\) channel blocker Ni\(^{2+}\) (100μM), with 96±3% (N=3, n=254, P=0.34) and 98±1% (N=3, n=138, P=0.95) of cells, respectively, producing criterion responses compared with normalised controls. However, the number of cells responding to either agonist was strongly suppressed by the presence of caffeine (10mM). The response to CCh was reduced to 4±2% (N=6, n=448, P<0.01) of that seen in controls, whilst the UTP response was reduced to 7±6% (N=5, n=160, P<0.01) of the control value (Figure 3.18). The results indicate that \([Ca^{2+}]_i\) transients evoked by purinergic and muscarinic stimulation arise from the release of \(Ca^{2+}\) from intracellular stores rather than from the entry of \([Ca^{2+}]_o\) via VGCCs.

The responses to GABA and glutamate were strongly suppressed by Ni\(^{2+}\); the number of cells responding was reduced to 1.5±1% (N=4, n=244, P=0.01) and 20±2% (N=3, n=235, P<0.04), respectively, of that seen in controls (Figure 3.18), while caffeine was without effect with 89±7% (N=3, n=171, P=0.37) and 111±7% (N=3, n=176, P=0.29)
of cells producing criterion responses. Furthermore, GABA and glutamate failed to evoke responses when retinas were perfused with low [Ca$^{2+}$] solution (N=3 for each, data not shown). Taken together, the experiments described here show that the [Ca$^{2+}$] response produced by GABA and glutamate largely result from Ca$^{2+}$ entry via VGCCs, as has been shown for whole chick retinas at times from E3 onwards (Yamashita et al., 1993; Sugioka et al., 1998; Catsicas and Mobbs, 2001).

3.3.16 The muscarinic and purinergic responses come from both mitotic and interphase cells

The relationship between the mitotic status of cells in the VZ at E6, and their responses to neurotransmitters, was examined using preparations co-labelled with Hoechst 33342 and Fluo-4 AM. Responses to muscarinic and purinergic agonists arose equally from the mitotic and the interphase cell populations; 50μM CCh evoked responses in 93±4% (N=7, n=213) of mitotic and 95±4% (N=7, n=260) of interphase (Figure 3.19) cells whilst 50μM ACh stimulated 68±11% (N=3, n=98) and 74±8% (N=3, n=111), respectively (data not shown). For 50μM UTP, the figures were 9±6% of mitotic and 13±4% of interphase cells (Figure 3.19). The number of cells responding to purinergic stimulation is much greater at E4 (see section 3.3.18), but the proportion of mitotic (97±3%, N=12, n=357) responses compared with interphase (98±2%, N=12, n=523) responses is very similar (Figure 3.19, inset).

3.3.17 The GABAergic and glutamatergic responses come from a predominantly interphase population

In contrast, to the cells responding to muscarinic and purinergic stimulation, the majority of cells responding to GABA and glutamate belonged to the interphase population of small cells. For GABA, 2±0.5% (N=4, n=120) of mitotic, and 33±8% (N=4, n=150) of interphase, cells responded, while for glutamate the figures were 9±3% (N=4, n=132) and 30±10.5% (N=4, n=150), respectively (Figure 3.19). Application of the AMPA/kainate agonist AMPA evoked responses in similar proportions of cells as glutamate: 9±2% (N=3, n=132) and 31±1% (N=3, n=150) of mitotic and interphase cells responded respectively. That the blockade of GABA$_A$ receptors affected only
interphase cells suggests that these receptors are expressed mainly by NDCs. In order to
determine whether or not the cells responding to GABA do indeed come from a
population of differentiated neurons, the cells responding to GABA were compared to
those expressing neuron-specific β-tubulin; the \([\text{Ca}^{2+}]_i\) responses to GABA were imaged
and the retinæ then fixed using 4% PFA in PBS. The retinæ were left \textit{in situ} in the
recording chamber during immunostaining for TuJ-1. The same region of retina imaged
during the GABA application was re-located and the cells examined for the presence of
TuJ-1\(^+\) labelling. Of the cells that responded to GABA in the one retina in which this
technically challenging experiment was completed, 76% (n=45) were TuJ-1\(^+\) and none
were in mitosis (n=20) (Figure 3.20). Repeated attempts to carry out the same
experiment for glutamate failed due to problems in registration of the two sets of
images.

The absence of a GABA-evoked \([\text{Ca}^{2+}]_i\) response in the mitotic population could also be
explained if GABA receptors were present on the cell but VGCCs were not. To
determine whether or not E6 PCs have VGCCs, Krebs’ solution containing high \([\text{K}^+]\)
(20mM) was applied to some retinae previously exposed to GABA. The majority of
both interphase and mitotic cells demonstrated a large increase in fluorescence upon
application of high \([\text{K}^+]\) suggesting that most PCs do indeed possess VGCCs.

3.3.18 Developmental change in sensitivity to neurotransmitter stimulation

In order to investigate whether or not the pattern of agonist sensitivity varied during
development, the drugs CCh, UTP, GABA and glutamate were applied to E4 and E5
retinæ and compared with the responses in E6 retinae. Retinæ prior to E4, and
subsequent to E6 were not tested.

All E4 retinae showed increases in \([\text{Ca}^{2+}]_i\) in response to 50 and 100µM CCh (N=25).
The proportion of VZ cells that responded to CCh (100µM) at E4 and E5 were 98±2\%\(^3\)
(N=11, n=845) and 96±3\% (N=5, n=250), respectively, whilst 99±0.4\% (N=7, n=365)

\(^3\) The percentages given in section 3.3.18 include responses from both mitotic and interphase cells. See
Figure 3.21 for details of the distribution of responses between these two populations.
of cells responded at E6. In contrast, the response to UTP (100µM) shows a significant developmental change. Unlike at E6, where only 11±5% (N=4, n=365) of VZ cells responded to UTP, 87±6% (N=12, n=880; P<0.01 compared with E6) of cells responded at E4 and 74±2% (N=4, n=160) of cells responded at E5. Roughly equal numbers of mitotic and interphase cells responded to CCh and UTP at all times tested. At both earlier and later times the responses to both agonists were oscillatory.

In contrast to the effects of UTP at E4, relatively few cells responded to GABA and glutamate (both 100µM) at this time (Figure 3.21). Only 8±3% (N=8, n=385) of cells showed [Ca^{2+}]_i changes greater than 10% ΔF/F in response to GABA compared with the 35±8% (N=6, n=320) responding at E6. Similarly, glutamate evoked responses in only 4±2%, (N=6, n=385) of the population whereas 39±11% (N=6, n=280) responded at E6. At E5, 13±2%, (N=6, n=300) and 15±2%, (N=4, n=160) of cells respond to GABA and glutamate, respectively. The kinetics of the GABA and glutamate evoked responses at each age consisted of single, monotonically declining transients. A larger proportion of interphase cells responded to these two agonists than mitotic cells at all times tested.

3.3.19 VZ cells can respond to more than one neurotransmitter

The data presented so far in this chapter shows that the cells within the E6 chick retinal VZ are able to respond to purinergic, muscarinic, GABAergic and glutamatergic stimulation and that the responses to the latter two agonists comes from a predominantly interphase population. The experiments in this section examine whether or not individual cells are capable of responding to more than one neurotransmitter. CCh, UTP, GABA and glutamate (all 100µM) were applied to Fluo-4-loaded retina from both E4 (N=4) and E6 (N=5) embryos, and the proportion of VZ cells showing [Ca^{2+}]_i responses determined. Retinae were sequentially exposed to CCh, UTP, GABA and glutamate. The order of application of the different drugs was changed with each new retina.
Cells of the E4 VZ respond to both muscarinic and purinergic stimulation

At E4, 93±5% (N=4, n=365) of VZ cells responded to bath application both of UTP and of CCh (both 100μM), suggesting that muscarinic and purinergic receptors may be co-expressed on cells in the VZ at this age (Figure 3.22a). Only 5±4% of cells responded to UTP alone, and 2±2% responded only to CCh. All cells responded to at least one of these two agonists. Application of either GABA or glutamate (both 100μM) evoked responses in less than 8±3% and 4±2% of E4 VZ cells, respectively. Of these, all also responded to CCh and most were in interphase. None of the cells responding to either GABA or glutamate were stimulated by UTP.

Cells of the E6 VZ respond to muscarinic, glutamatergic and GABAergic stimulation

The ability of ventricular cells to respond to more than one neurotransmitter was also tested at E6, a time when GABA and glutamate responses are evoked in a greater proportion of cells than at E4. Only 8±5% (N=5, n=385) of cells responded to UTP and these also responded to CCh, but not to GABA or glutamate. CCh, GABA and glutamate evoked responses in 88±5%, 38±9% and 28±4% (N=5, n=385) of the cells tested respectively. Only 5% of the cells in the VZ showed [Ca^{2+}]_i changes in response to all three agonists (Figure 3.22b). However, 8±2% of mitotic and 20±1% of interphase cells were able to respond to both the application of CCh and that of glutamate. Experiments described in section 3.3.17 showed that only ~2% mitotic cells respond to GABA and that in a preparation that was co-labelled with TuJ-1, none of the 20 mitotic, TuJ-1^- cells responded. In congruence with those results, only 2±2% of the mitotic population responded to both GABA and CCh. GABA failed to evoke any response from the mitotic population in 3 of the 5 retinae examined. GABA stimulates a much larger proportion of the interphase (36%) than the mitotic population, and all but 10 of the 153 interphase cells analysed that responded to GABA also responded to CCh. At E6, GABA and glutamate evoke responses from about a third of cells within the VZ (see section 3.3.17), but only 2.5±2% (0.4% mitotic and 4.5% interphase cells) of the population responded to both glutamate and GABA at this age.
Thus, individual cells in the VZ of the early embryonic retina are able to respond to more than one neurotransmitter. This suggests that VZ cells express multiple receptor types. Since the pattern of sensitivity to different agonists changes from E4 to E6 it is likely that the expression of these receptors is developmentally regulated. An alternative explanation involving the release of endogenous neurotransmitters in response to exogenous application of agonists is explored in the discussion.

3.3.20 Comparison of the relative effectiveness of purinergic and muscarinic agonists in evoking [Ca^{2+}]_{i} responses

Section 3.3.16 demonstrated that purinergic and muscarinic agonists can induce changes in [Ca^{2+}]_{i} in both mitotic and interphase cells in the early embryonic retinal VZ. The responses to both agonists are often oscillatory and involve the release of Ca^{2+} from intracellular stores (see section 3.3.15). The characteristics of the responses to UTP and CCh were further examined to assess whether or not there is a systematic difference in the [Ca^{2+}]_{i} responses evoked by these two agonists. 1µM UTP and 10µM CCh each evoke responses in ~50% of the population of cells in the VZ. However, application of 1µM UTP consistently evoked larger [Ca^{2+}]_{i} responses than 10µM CCh. Individual cells showed an average change in fluorescence (ΔF/F) of 58±9\% (N=4, n=68) in response to UTP compared with 31±4\% (N=4, n=80) following the application of CCh (Figure 3.23). Both agonists produced responses of similar magnitude in both mitotic and interphase cells. The frequency of oscillations in the responses to UTP and CCh was concentration dependent. At low concentrations of either agonist the response was often a single peak while at higher concentrations the drugs produced several peaks within the same period of application. Interestingly, while the amplitude of the response to 10µM CCh was typically smaller than the response to 1µM UTP, the frequency of oscillations in response to CCh was higher. The mean number of oscillations evoked by a single 2min application of 10µM CCh and 1µM UTP were 3.5±1 (N=4, n=100) and 2.4±0.07 (N=4, n=229, P<0.01), respectively.
3.4 Discussion

3.4.1 Confocal Ca\(^{2+}\) imaging in the developing VZ

A growing body of evidence suggests that, besides their role as molecules mediating communication between nerve cells in the mature nervous system, neurotransmitters play important roles during neuronal development, even at times prior to synaptogenesis. Studies show that, prior to synapse formation at E12 (Hughes and LaVelle, 1974), a number of neurotransmitter receptors are thought to be expressed by embryonic chick retinal cells; responses have been observed following the exogenous application of ACh, GABA, glutamate and purine nucleotides and these responses are developmentally regulated (Yamashita and Fukuda, 1993a, b; Yamashita et al., 1994; Bonness, 1998; Sakaki et al., 1996; Sugioka et al., 1996; Sugioka et al., 1998). There is also evidence to suggest that cells are capable of releasing neurotransmitters at these early stages. Components of the exocytosis mechanism, such as synaptotagmin and syntaxin, are expressed in the chick retina as early as E4 (Bergmann et al., 2000). Release of ACh, GABA, ATP and glutamate has been demonstrated in cultures of chick retinal cells and retinal organ cultures (Bonness, 1998; Duarte et al., 1996; Sugioka et al., 1999; Santos et al., 1999). GABA and glutamate receptors are found early in the development of the cortex (LoTurco et al., 1995; Fiszman et al., 1999; Haydar et al., 2000) and the endogenous release of both GABA and glutamate has been implicated in the regulation of cell proliferation and migration there (Komuro and Rakic, 1993; LoTurco et al., 1995; Haydar et al., 2000).

The use of confocal imaging allows \([Ca^{2+}]\), responses to be attributed to individual cells. The experiments presented here demonstrate that spontaneous and neurotransmitter-evoked \([Ca^{2+}]\) activity occurs in cells of the early embryonic chick retinal VZ. The comparison of the \([Ca^{2+}]\) responses of cells together with the staining pattern of their chromatin and post-hoc immunostaining for the neuronal marker \(\beta\)-tubulin indicates two findings; firstly, the retinal VZ includes both PCs and a significant proportion of NDCs and secondly,
different receptors may be expressed by the proliferating and differentiating populations and that this expression changes with time.

Imaging throughout the thickness of the retina showed that cells at all depths between the ventricular and the vitreal surfaces displayed spontaneous \([\text{Ca}^{2+}]_i\) transients. The time-scale of these transients varied markedly. In many, the onset and decay was rapid, reminiscent of those observed in the VZ. These events encompassed the whole cell, including processes. In others, the onset was slower and in some cases it was possible to follow the propagation of a \(\text{Ca}^{2+}\) wave within the cell. These transients could travel in either direction, propagated at speeds of \(\sim 10\mu\text{m/s}\), and lasted for tens of seconds. \([\text{Ca}^{2+}]_i\) waves propagate between Müller cells at similar speeds (23\(\mu\text{m/s}\), Newman and Zahs, 1997). These speeds are also consistent with calculated velocities of global IP\(_3\) and CICR-dependent \([\text{Ca}^{2+}]_i\) waves described in several other cells types (e.g. Thomas et al., 1996; for review, see Rottingen and Iversen, 2000). Such intracellular waves are thought to occur when cells are stimulated to produce IP\(_3\) through the action of agonists or some other stimulation. It is possible that some of the activity observed here in the retina may represent endogenous activation of neurotransmitters receptors or some other membrane receptor.

3.4.2 Endogenous release of neurotransmitter drives spontaneous \([\text{Ca}^{2+}]_i\) activity in retinal VZ cells

Spontaneous changes in \([\text{Ca}^{2+}]_i\) have been examined in immature neurons and PCs in the developing cortex (Owens et al., 1996; Owens and Kriegstein, 1998; Owens et al., 2000) and during, and subsequent to, the period of synaptogenesis in the developing retina (Catsicas et al., 1998; Wong et al., 1998). The experiments described above show that spontaneous \([\text{Ca}^{2+}]_i\) activity is present in retinal VZ cells during the first week of development. This activity mostly takes the form of transients within individual cells although pairs and larger groups of cells sometimes undergo co-ordinated activity. This wave-like spread of \([\text{Ca}^{2+}]_i\) signals between cells is discussed in Chapter 6. Comparison of the changes in \([\text{Ca}^{2+}]_i\) with the pattern of chromatin in cells in the VZ showed that spontaneous \([\text{Ca}^{2+}]_i\) events occurred in both interphase cells and those in the process of mitosis. This activity was unaffected by TTX, and thus appears to be independent of
action potential production, as in the cortex (Owens and Kreigstein, 1998; Owens et al., 2000). However, in contrast to the findings of Owens and Kreigstein, \([Ca^{2+}]_i\) activity in the retinal VZ depends upon transmitter release. Muscarinic and purinergic antagonists caused significant reductions in the frequency of spontaneous events in both the mitotic and interphase cell populations. Markers of cholinergic activity including ChAT and AChE are present at the times investigated (this chapter and Layer et al., 1987; Layer, 1991). Increasing endogenous levels of BuCh and ACh by application of butyryl- and acetyl-cholinesterase inhibitors greatly enhanced the level of spontaneous activity. Eserine has been noted to have a direct effect at the nAChR (Shaw et al., 1985; Okonjo et al., 1991). However, this is unlikely to account for the effects of eserine here since functional nAChRs are present in only small numbers of cells at this time (see below). Apyrase, which reduces the concentration of extracellular purinergic nucleotides, caused a significant reduction in the number of spontaneous \([Ca^{2+}]_i\) events. These results indicate that endogenous activation of muscarinic and purinergic receptors occurs in situ and leads to changes in \([Ca^{2+}]_i\). In contrast to the cortex, where spontaneous \([Ca^{2+}]_i\) activity is independent of amino acid neurotransmitters, glutamatergic and GABAergic antagonists also decreased the number of spontaneous events. However, these effects were more prominent in the interphase population of cells in the retina.

### 3.4.3 Muscarinic responses in embryonic chick retina

ACh causes oscillatory changes of \([Ca^{2+}]_i\) in ventricular cells in the early embryonic chick retina. The retinal response to ACh was blocked by the mAChR antagonist atropine, and the mAChR agonists CCh and muscarine induced responses very like those produced by the presumed endogenous agonist, ACh. CCh can act with low affinity at the nAChR (A. Gibb, personal communication) and in situ hybridisation studies have detected mRNA for nAChR receptor subunits in the chick retina at E4.5 (Hamassaki-Britto et al., 1994). However, application of nicotine at this time and at E6 produced responses in only a very small number of VZ cells (~6%), indicating that functional nAChRs are expressed in very low numbers prior to E7. In contrast, CCh and muscarine exert powerful effects on \([Ca^{2+}]_i\) within VZ cells at this time. Stimulation of mAChRs has also been shown to increase \([Ca^{2+}]_i\) in the VZ of the developing rabbit
retina, whilst nicotinic responses arise from the immature RGC and amacrine cell populations (Wong, 1995).

Four of the 5 mammalian mAChR subtypes have avian homologues (cm2-5) and mRNA and protein of the cm2, cm3 and cm4 subtypes have been located in embryonic retinal tissue (McKinnon and Nathanson, 1995). Both the cloned cm2 and cm4 subtypes demonstrate an anomalously high affinity for pirenzipine (Tietje et al., 1990, 1991) and the cm4 subtype also shows moderate affinity for AFDX-116 (Tietje et al., 1990). Since the response to CCh observed here in the chick retinal VZ was only partially blocked by the M₂ antagonist, AFDX-116, but completely inhibited by both the M₁ antagonist pirenzipine and the M₄ antagonist, tropicamide, the early muscarinic response is likely to be mediated primarily via the M₄ receptor. This conclusion is supported by immunoprecipitation and immunoblot analyses, which show that during early development the cm4 subtype predominates whilst expression of cm3 and cm2 increases during the second week of development (Nadler et al., 1999). The pharmacological studies carried out in this chapter were based on population responses and cannot rule out the expression of other receptor subtypes in smaller numbers, either within the same cells that express the cm4 receptor or in a separate sub-population.

3.4.4 Purinergic responses in embryonic chick retina

ATP produced oscillatory changes in \([Ca^{2+}]_i\) in cells in the chick retinal VZ via stimulation of P2 receptors. Purinergic responses have been observed in the whole retina (Sakaki et al., 1996; Sugioka et al., 1996). The pharmacological characterization of P2 antagonists has been confounded by the use of different tissues, species, assay systems and their ability to inhibit the actions of endogenous ATPase activity, thereby potentiating the effects of endogenous ATP (for review, see Burnstock, 1999). While the pharmacological identification of purinergic receptor subtypes is complex, some tentative suggestions as to the nature of receptors found in the chick VZ can be made on the basis of the effect of the agonists and antagonists used in the experiments described above. Whilst specific P2X-receptor agonists were not tested in the experiments described above, the involvement of these receptors here is unlikely given that UTP, an agonist with reasonable selectivity for P2Y receptors, was markedly more effective than ATP. Furthermore, previous studies in the E3 to E5 chick retina have also shown that
the P2X agonist α,β-methylene ATP has no effect on [Ca^{2+}]_i (Sugioka et al., 1996). The order of agonist potency in the E4 chick retina, assessed on the basis of the number of cells that responded, was UTP=ADP>ATP>UDP. The response to UTP was blocked to a significant extent by suramin, Reactive Blue and PPADS. In contrast to the P2Y1 subtype, which is specifically activated by adenine nucleotides (Schachter et al., 1996), the P2Y2, P2Y4 and P2Y6 subtypes are all activated by uridine nucleotides with different sensitivities to adenine nucleotides. The mammalian P2Y6 is strongly activated by UDP (Nicholas et al., 1996a,b). Purinergic antagonists are poorly selective, acting on more than one subtype. However, the P2Y2 subtype is insensitive to Reactive Blue whilst suramin has little effect at the P2Y4 receptor (Charlton et al., 1996). PPADS has been shown to block P2Y1, P2Y4 and P2Y6 receptors, although its actions at the P2Y4 receptor are disputed (see section 1.6.2 and Communi et al., 1996; Charlton et al., 1996). The cloned chick p2y3 receptor (Webb et al., 1996) was initially thought to be a novel P2Y receptor. However, recent pharmacological studies indicate that it is the avian homologue of the P2Y6 receptor (Li et al., 1998). UTP and UDP act as full agonists at this receptor although the chick variant also demonstrates significant sensitivity to ADP with the precise agonist potency order depending upon the cell type in which the cloned receptor is expressed (Webb et al., 1996). For example, the recombinant p2y3 receptor, has an agonist potency order of ADP>UTP>ATP>UDP when expressed in Xenopus oocytes (Webb et al., 1996) but when expressed in Jurkat cells the order is UDP>UTP>ADP>ATP (see Li et al., 1998). Boyer et al. (1997) have also cloned an avian homologue of the mammalian P2Y4 receptor. In contrast to the mammalian subtype, which is selectively activated by the tri-nucleotide UTP but not ATP, both ATP and UTP stimulate the novel avian P2Y4 receptor. It is quite possible that more than one subtype mediates the purinergic responses described here. One or both of the avian P2Y4 and P2Y3 receptors are candidates for mediating the purinergic response in the early embryonic chick retina. However, the potency of UTP and ADP and the block by suramin, point more strongly towards the P2Y3 receptor. Further, dinucleotides may be the active compounds mediating the observed response to UTP, since endogenous ecto-nucleotidases can hydrolyse the trinucleotide, and ecto-nucleoside diphosphokinases can interconvert di- and tri-phophates. Both families of protein are found in a variety of tissues, including the brain (for review, see Zimmerman, 2000). The lack of commercially available selective receptor antagonists
makes it impossible to determine, in this experimental preparation, whether another subtype is expressed alongside the P2Y3 receptor. Immunohistochemical or in situ hybridisation studies could provide more conclusive evidence regarding the subtypes involved. There are, however, few commercially available antibodies and none have proven specificity for chick. Attempts to label embryonic chick tissue using P2Y1, P2Y2, and P2Y4 antibodies have produced inconsistent results (J. Gale, N Luneborg, personal communications).

Raised [Ca\(^{2+}\)] can result from receptor activation leading to membrane depolarisation and the consequent activation of VGCCs, the direct activation of Ca\(^{2+}\)-permeable receptors or the activation of receptors coupled to the production of 2\(^{nd}\) messengers that stimulate Ca\(^{2+}\) release from intracellular stores. The muscarinic and purinergic [Ca\(^{2+}\)] responses observed in the early chick retina predominantly depend upon the release of Ca\(^{2+}\) from intracellular stores. The number of cells responding to CCh and UTP were unaffected by the non-selective Ca\(^{2+}\) channel blocker Ni\(^{2+}\) or by removal of extracellular Ca\(^{2+}\). This is supported by experiments in the whole chick retina in which the VGCC blocker nifedipine and low external Ca\(^{2+}\) had little effect on the amplitude of either the muscarinic-evoked or the purinergic-evoked Ca\(^{2+}\) signal (Yamashita et al., 1994; Sugioka et al., 1996). These results indicate that the responses are mediated by Ca\(^{2+}\) release from intracellular stores, a conclusion supported by the findings described here showing that purinergic and muscarinic responses were significantly attenuated by pre-application of caffeine, which causes the emptying of intracellular Ca\(^{2+}\) stores. The oscillatory nature of the responses is also typical of intracellular Ca\(^{2+}\) release (for review, see Berridge, 1993). In contrast, muscarinic responses in the developing rabbit retina are abolished both by removal of extracellular Ca\(^{2+}\) and the presence of high concentrations of Ni\(^{2+}\) (1-5mM) (Wong, 1995). Wong proposed that the atypical muscarinic response observed in the rabbit retina is a result of Ca\(^{2+}\) influx through receptor-operated channels rather than release from intracellular stores. The effects of Ni\(^{2+}\) on the rabbit muscarinic response require further investigation since there appears to be no further suggestion in the recent literature that muscarinic responses involve Ca\(^{2+}\) influx.

Sakaki et al. (1996) demonstrated that chick retinal [Ca\(^{2+}\)] responses to ATP and CCh were blocked by the PLC\(\beta\) inhibitor U-73122, which prevents the production of IP\(_3\), and
enhanced by pre-treatment with lithium, which prevents the hydrolysis of IP$_3$ in the rat brain (Berridge et al., 1982). Interestingly U-73343, an analogue of U-73122, inhibited the response to CCh but was without effect on the response to ATP (Sakaki et al., 1996). Thus, Ca$^{2+}$ is mobilized from intracellular stores in the response to both muscarinic and purinergic stimulation but the two receptors are likely to use different signal transduction pathways. ATP, UTP and CCh have all been shown to increase phosphoinositide turnover in retinal cultures and the intact chick embryo retina (Sanches et al., 2002). Whilst many mAChR subtypes are coupled to the G$_s$ G-protein, which stimulates PLC and IP$_3$ production, the mammalian M$_4$ receptor couples with the G$_{i/o}$ class of G-proteins, which inhibit adenylate cyclase and cause a reduction in cAMP levels. IP$_3$-induced rises in [Ca$^{2+}$], would therefore require the cm4 receptor to operate differently to its mammalian counterpart. Little is known about the signal transduction mechanisms of the avian muscarinic receptors but there is increasing evidence that the $\beta_y$ subunits of the G$_{i/o}$ G-proteins are capable of directly acting on PLC$\beta$ (for review, see Rozengurt, 1998). There is also extensive debate over whether IP$_3$-sensitive and caffeine/ryanodine-sensitive Ca$^{2+}$ stores represent independent sections of the endoplasmic reticulum Ca$^{2+}$ store, or whether the two are confluent (for review, see Blaustein and Golovina, 2001). The majority of neuronal types are believed to contain stores activated by both mechanisms (see Blaustein and Golovina, 2001; Bootman et al., 2001). Some investigators have further proposed that chick retinal cells contain only IP$_3$-insensitive Ca$^{2+}$ stores (Cristovao et al., 1997) or only caffeine-insensitive stores (Sakaki et al., 1996). As discussed above, drugs acting at either the “IP$_3$-sensitive” or “caffeine/ryanodine-sensitive” stores inhibit the response to muscarinic and purinergic stimulation. Assuming there are two independent Ca$^{2+}$ stores, the most likely explanation is that stimulation of these receptors causes an initial increase in IP$_3$, which releases Ca$^{2+}$ from IP$_3$-sensitive stores. This Ca$^{2+}$ release then feeds back on to the caffeine/ryanodine-sensitive stores causing CICR and the major increase in [Ca$^{2+}$]. This proposed mechanism of release would be in keeping with the inhibitory effects of blocking either mechanism of release and has been demonstrated in many other cell types (see Fagni et al., 2000). Further careful pharmacological dissection of the pathways involved will be required on isolated cells to establish the interplay between these Ca$^{2+}$ stores in the chick retina.
3.4.5 GABAergic responses in embryonic chick retina

GABA induces a monotonic rise in \([\text{Ca}^{2+}]_i\) in circa one third of cells in the VZ of the chick retina. These responses are completely blocked by the GABA_\text{A} receptor antagonist, bicuculline. GABA usually acts as an inhibitory transmitter in the adult CNS. GABA_\text{A} receptors couple to Cl\(^-\) permeable channels and receptor activation causes an influx of Cl\(^-\) and a consequent shift in the membrane potential towards the Cl\(^-\) equilibrium potential, thereby maintaining a hyperpolarized, and hence less excitable, state. In early development GABA_\text{A} receptor activation results in Cl\(^-\) leaving the cell, causing a depolarisation that activates VGCCs (see below). The high [Cl\(^-\)]\(_i\) is thought to be maintained by furosemide-sensitive Cl\(^-\) transporters (Nishi et al., 1974; Frambach and Misfeldt 1983; Reichling et al., 1994; see also Clayton et al., 1998). Zhang et al. (1991) suggest that the Cl\(^-\) reversal potential becomes more negative as the Cl\(^-\) extrusion system develops. Thus, the ability of GABA to cause increased [Ca\(^{2+}\)]\(_i\), via secondary activation of VGCCs would be expected to decrease alongside development of the extrusion mechanism. This is supported by the observation that GABA-evoked [Ca\(^{2+}\)]\(_i\) responses are absent in the chick retina after E14 (Allcorn, 1996; Catsicas and Mobbs, 2001).

3.4.6 Glutamatergic responses in embryonic chick retina

Application of glutamate caused monotonic elevations in \([\text{Ca}^{2+}]_i\) in circa one third of cells in the chick retinal VZ. Glutamate could act by directly activating Ca\(^{2+}\)-permeable NMDA receptors or by activating AMPA/kainate receptors. Activation of the AMPA/kainate receptors may cause Ca\(^{2+}\) entry via depolarisation and activation of VGCCs or Ca\(^{2+}\) may enter through the receptor itself (see below). The involvement of NMDA receptors is unlikely because the response to glutamate occurs at a time when others have shown the chick retina to be unresponsive to NMDA (Allcorn et al., 1996) and, as shown above, the response is not inhibited by application of AP5. In contrast, the AMPA/kainate receptor agonist AMPA evoked changes similar to those caused by glutamate, and the AMPA/kainate receptor specific antagonist, NBQX, blocked both AMPA and glutamate-evoked responses.
The increase in $[Ca^{2+}]$, following both GABAergic and glutamatergic stimulation is likely to be due to the opening of VGCCs. The non-specific Ca$^{2+}$ channel blocker Ni$^{2+}$ virtually abolished the response to GABA and markedly reduced the number of cells responding to glutamate. Neither agonist evoked $[Ca^{2+}]$, rises when Ca$^{2+}$ was removed from the extracellular solution. Conversely, depletion of intracellular stores by caffeine was without effect suggesting that CICR is not involved in the response to these agonists. Imaging studies in the whole retina have shown that the L-type Ca$^{2+}$ channel blocker, nifedipine, also prevents the GABA-evoked Ca$^{2+}$ response (Yamashita and Fukuda, 1993a; Catsicas and Mobbs, 2001). Ca$^{2+}$ may enter cells directly through Ca$^{2+}$ permeable forms of the AMPA/kainate receptor, which have been shown to be present in the chick retina by E6 (Allcorn et al., 1996). Ni$^{2+}$ can block AMPA receptors directly (Telgkamp et al., 1996), although it does so in a concentration dependent manner. The concentration dependence of the blockade of AMPA by Ni$^{2+}$ may explain why a small number of cells still respond to glutamate application when Ni$^{2+}$ is present.

3.4.7 Neurotransmitter responses change with age

The period of development investigated in this thesis covers the peak of cell proliferation and the initiation of neurogenesis of many retinal cell types (Prada et al., 1991). The expression of neurotransmitter receptors and the response to neurotransmitters change markedly during this period. Muscarinic receptors are likely to play an important role in the development of the retina since the majority of cells in the VZ respond to muscarinic agonists during early development (E4 to 6) and the response declines thereafter (data not shown). Yamashita et al. (1994) report a strong muscarinic response at E3 chick retinae, but smaller responses from E5 onwards. Large numbers of ventricular cells respond to UTP at E4, somewhat fewer at E5 and markedly less at E6. Sakaki et al. (1996) observed a similar developmental decrease in the Ca$^{2+}$ response to ATP in the whole chick retina. Previous imaging studies showing developmental changes in the size of Ca$^{2+}$ responses have received criticism as the observed decline in fluorescence signal could be due to increasing retinal thickness causing differential absorption of fluorescence. Alternatively, the decline could be explained by a decrease in the number of cells within the measurement area. The confocal studies reported here are unlikely to suffer from differential absorption of fluorescence since retinae were always positioned such that the VZ lay adjacent to the imaging lens. Furthermore,
results are expressed as a percentage of cells responding within a population, which removes the effect of a change in the number of cells in either the VZ or the retina as a whole. Reasons for the decline in the purinergic and muscarinic responses were not investigated but possible reasons include receptor down-regulation and decreased expression, and a functional change in the receptor-effector coupling of mAChRs to IP$_3$ metabolism and Ca$^{2+}$ mobilisation (Calvet and Ventura, 1995).

In contrast to the decline in the purinergic response between E4 and E6, responses to GABA and glutamate increased over the same period. At E4, small numbers of cells responded to GABA and very few to glutamate. Responses to both agonists progressively increased over the next two days. Other studies have shown that the [Ca$^{2+}$]$_i$ responses of retinal cells to both GABA and glutamate peak at times much later than the period studied here (around E8 and E12 respectively; Allcorn, 1996; Allcorn et al., 1996) suggesting developmental roles that are distinct from those mediated by purinergic and muscarinic receptors.

3.4.8 The distribution of neurotransmitter responses within the interphase and mitotic cell populations

[Ca$^{2+}$]$_i$ responses to GABAergic and glutamatergic stimulation largely arise from a population of cells that are in interphase. Post-hoc labelling for the neuronal marker β-tubulin with TuJ-1 indicates that the GABA [Ca$^{2+}$]$_i$ response arises largely from a population of NDCs. In studies of the rat neocortex, whole-cell electrophysiological recordings of clusters of VZ cells indicated that they could respond to GABAergic stimulation (LoTurco et al., 1995). GABA-evoked depolarisation was absent at the onset of neurogenesis (~E12) but rose to include ~30% of cells over the next three days and further still by E16. LoTurco and Kriegstein did not attempt to identify the cells responding and so these figures could include responses from the population of NDCs. Another possible explanation for the apparent discrepancy between the electrophysiological recordings made by LoTurco et al. (1995) in the cortex, and those obtained here by Ca$^{2+}$ imaging of the chick retina is that GABA$_A$ receptors are present in retinal PCs, but are not yet effectively coupled to VGCCs, which would prevent the expected Ca$^{2+}$ influx. However, this explanation is unlikely since [K$^+$]-
induced depolarisation evoked large changes in \([\text{Ca}^{2+}]_i\) in the majority of retinal PCs, indicating that they do express functional VGCCs during the first week of development. It is conceivable that GABA\(_A\)-receptors are expressed by PCs in both tissues but only during the final cell cycle, prior to terminal division. Marie et al. (2001) proposed that, in the cortex, the functional response to GABA is correlated with neuronal differentiation. Changes in membrane potential and \([\text{Ca}^{2+}]_i\) were confined to cells undergoing differentiation; only a very small fraction of the response arose from cells identified as neural PCs. Within this PC fraction, some demonstrated membrane depolarisation but virtually none underwent a correlated change in \([\text{Ca}^{2+}]_i\). In contrast, \(\sim90\%\) of the NDCs in the cortical plate were depolarised by GABA and \(\sim55\%\) showed a corresponding increase in \([\text{Ca}^{2+}]_i\). If the timing of GABA\(_A\) receptor expression is the same in the retina as in the cortex, the fact that \([\text{Ca}^{2+}]_i\) changes due to GABA were rarely seen in retinal PCs may not be surprising. Combined electrophysiological recordings and labelling of cells with either PC markers or early markers of differentiation will be required to establish when during terminal division or differentiation, GABA is able to induce membrane depolarisation. Alternatively, the different distribution of responses to GABA in the retina and the neocortex may reflect genuine differences in the role of this neurotransmitter in the development of these two tissues.

As with GABA, the responses to glutamate arise primarily from the interphase population in the chick retinal VZ and thus the expression of glutamate receptors may also be restricted to the differentiating population. Unfortunately, attempts to combine \(\text{Ca}^{2+}\) imaging with TuJ-1 immunostaining were unsuccessful. A small but potentially significant proportion of mitotic cells did respond to glutamate and thus glutamate receptors appear to be expressed by at least some PCs. The cells that respond may be a population of PCs in the final cell cycle, prior to terminal division, that show precocious expression of the receptor. Whole-cell patch clamp recordings made in the rat neocortex indicate that glutamate receptors are expressed at or around the time of terminal division and exit from the proliferating cell cycle (Maric et al., 2000). Inward currents and changes in \([\text{Ca}^{2+}]_i\) were only seen in differentiating neurons. However, a small proportion of cells stained positive for both BrdU and the neuronal marker TuJ-1, and these cells also responded to glutamate. Thus, glutamate receptors appear to be expressed at or very close to the time of terminal division in the neocortex. Such
precocious expression of receptor proteins is perhaps not surprising since in the retina other markers of differentiation, such as the RGC marker RA4, are expressed during the final cycle. RA4 protein is expressed during the late stages of mitosis and RA4 mRNA is present during G2 of the final cell cycle (McCabe et al., 1999). It seems likely that glutamate receptors are expressed by PCs at some stage in the final cell cycle although further studies, using electrophysiological recordings combined with immunocytochemistry, will be required to confirm this hypothesis.

In contrast to GABA and glutamate, which produce responses in smaller, and largely interphase, populations of cells, responses to muscarinic stimulation are almost ubiquitous from E4 to E6, suggesting that muscarinic receptors play important roles in both NDCs and PCs at this time. That the vast majority of cells in the E4 VZ demonstrated changes in [Ca^{2+}]i in response to both muscarinic and purinergic agonists suggests that all PCs express these receptors. However, it is possible that VZ cells are linked to cells expressing the receptors by gap junctions, which would allow the Ca^{2+} signal to spread between cells (LoTurco and Kriegstein, 1991b; Chapter 6, this thesis) or that muscarinic and/or purinergic stimulation acts on a receptor-bearing subpopulation, evoking the release of some other factor which acts on the rest of the VZ cell population. Gap junction coupling between retinal PCs is described in Chapter 6 and thus signal-spread via this mechanism remains a possibility. Whether muscarinic and/or purinergic stimulation bring about the release of a second stimulating factor remains an interesting issue to be resolved. Purinergic responses are widespread at E4, when the majority of cells are actively proliferating (Kahn, 1974; Prada et al., 1991) and few have undergone differentiation (Nishimura, 1980; Prada et al., 1991). The response then declines rapidly between E4 and E6. Interestingly, cells that responded to purinergic stimulation never responded to either GABA or glutamate, and vice versa. The time course of the [Ca^{2+}]i response to UTP (and ATP) described above bears an inverse relationship to the time course of neurogenesis (Prada et al., 1991). The decrease in the proliferative activity of PCs may, therefore, be related to the reduction in purine-inducible Ca^{2+} mobilization. This possibility is considered further in Chapter 4.
3.5 Further studies

The experiments presented here have shown that cells in the retinal VZ respond to purinergic, muscarinic, GABAergic and glutamatergic stimulation. The pattern of receptor expression appears to change with age and the receptors are differentially distributed between PCs and NDCs. The use of electrophysiological techniques could provide further information regarding the expression and later down regulation of these receptors in VZ cells. *In situ* hybridisation combined with detailed pharmacological investigations will be required in order to identify the receptor subtypes involved in mediating the muscarinic and purinergic responses described in this chapter. The imaging experiments presented clearly show that at early times most ventricular cells are competent to respond to both these neurotransmitters. However, it is not possible to conclude whether all cells express one or both receptors or whether following stimulation, a subpopulation of cells transmit some signal to their neighbours. Immunocytochemical studies performed on isolated PCs would permit assessment regarding whether PCs co-express mAChRs and P2 receptor.

3.6 Summary

The results presented in this chapter demonstrate the presence of spontaneous \([Ca^{2+}]\), activity in chick retinal VZ cells. Since it is unaffected by TTX, this activity is independent of Na+-dependent action potentials, as it is in the cortex (Owens and Krieeigstein, 1998). However, in contrast to the cortex, \(Ca^{2+}\) calcium activity in the retinal VZ depends upon transmitter release. Comparing the changes in \([Ca^{2+}]\), with the pattern of chromatin shows that GABAergic and glutamatergic receptors are present within a largely interphase population of VZ cells. Combined \(Ca^{2+}\) calcium imaging, chromatin staining and immunocytochemistry for neuronal tubulin further suggest that GABA\(_A\)-Rs are mainly expressed by a population of NDCs. In contrast, mAChRs are almost ubiquitous at E6, suggesting that these receptors play important roles in both NDCs and PCs at this time. The expression of transmitter receptors by PCs changes with time; while most mitotic and interphase cells respond only to CCh, and not UTP, at E6, the vast majority of cells respond to both agonists at E4. The functional significance of this change in the pattern of response with time is unknown, but it is possible that it correlates with a switch from symmetrical division of PCs at early times in development, to increased numbers of asymmetric divisions (Desai and McConnell, 2000). Together with the fact that the responses to GABA and glutamate appear to arise from an interphase, and potentially differentiated, population, this raises the hypothesis that purinergic and muscarinic transmitters are in a position to act on the PC mitotic cycle, whilst GABA and glutamate would be without effect. The effects of these transmitters on the proliferative cycle are investigated in Chapter 4.
Figure 3.1. VZ cells can be labelled with both Fluo 4-AM, to measure their [Ca^{2+}],
responses, and Hoechst 33342 to determine their mitotic status. A, In Fluo-4 (10μM) labelled preparations two distinct populations of cells can be identified within the VZ. Large dark cells (arrows) and smaller, more brightly labelled ones (arrow heads). B, The same region shown in A, labelled with Hoechst 33342 (2μM). The large profiles contain condensed chromatin and are mitotic (arrows). The chromatin within the nuclei of the brightly labelled, and smaller, cells is typical of cells in interphase (arrow heads). C, The VZ of an E6 retina labelled with TuJ-1 (red), an antibody for neuron-specific tubulin, and Hoechst 33342 (green). Several cells in interphase cells are TuJ-1^+ (see results). D, The GCL and developing fibre layer (arrows) of an E6 retina labelled with TuJ-1 (red) only. Nearly all cells are TuJ-1^+. Scale bars 5μm.
Figure 3.2. Spontaneous $[\text{Ca}^{2+}]_i$ transients in E6 VZ cells. 

A, Examples of spontaneous changes in $[\text{Ca}^{2+}]_i$ ($\Delta F/F$) in individual VZ cells, at 36°C. In any given cell, events occurred at a rate of $\sim 1$ transient/250s ($n=99$, $N=3$) and had durations of 13.8±1.3s. Processes of other neuroepithelial cells terminate at the VZ and appear as small profiles <1μm diameter (arrow heads). These also demonstrate changes in $[\text{Ca}^{2+}]_i$ (Aii, arrow heads). 

B, The duration of spontaneous events was measured as the time ($t_{50}$) spent above half maximum fluorescence. In order to assess the time course of spontaneous events images were taken every 0.9s. 

C, A paired event occurring between cells (1 and 2) prior to cytokinesis. Top panel, Hoechst 33342 labelling showing the state of the chromatin in cells 1 and 2. 

D, Transients may spread to invade several cells. Seven interphase (1-7) cells are highlighted in the top panel (Hoechst 33342 labelling) and the change in $[\text{Ca}^{2+}]_i$, plotted in the traces (below). The $[\text{Ca}^{2+}]_i$ transient was initiated in cell 4 and spread as a wave into the surrounding cells. Scale bar: 5μm.
Figure 3.3. Spontaneous \([\text{Ca}^{2+}]\) transients occur throughout the depth of the neural retina. A, ‘virtual z scan’ of Fluo-4 AM labelled E6 retina. Asterisks indicate cells that showed spontaneous \([\text{Ca}^{2+}]\) transients during the 500s imaging period. Scale bar: 10\(\mu\)m Five cells have been highlighted and the changes in \([\text{Ca}^{2+}]\), occurring in these are shown in the traces in B. Images were acquired at 0.25Hz.
Figure 3.4. Propagation of [Ca\textsuperscript{2+}] transients in cells of the E6 retina. A, Fast [Ca\textsuperscript{2+}] \textit{ii} transients cause changes in [Ca\textsuperscript{2+}] at all regions along the length of the cell in <1 sec; \textit{ii}, 'virtual z-scan' of a Fluo-4 AM labelled retina in which a single cell has been highlighted. The transient is initiated near ROI 1 and propagates to ROI 3 (44\,\mu m) between 2 scans. \textit{ii}, traces of changes in \Delta F/F at 3 ROIs along the length of the cell. [Ca\textsuperscript{2+}] increases above criterion at rates faster than the time resolution of the microscope (0.98 s/scan). Note the top scale bar in A\textit{ii} refers to ROI 1 whilst the lower scale bar applies for ROIs 2-5. B, Slow [Ca\textsuperscript{2+}] \textit{ii} transients propagate along the length of a PC; \textit{i}, a 'virtual z-scan' of a Fluo-4 AM labelled E5 retinae. A spontaneous transient starts at the ventricular end of a PC (outlined) and spreads up to the vitreal end. \textit{ii}, traces 1-5 show changes in \Delta F/F acquired at 1 Hz of 5 ROIs along the length of the cell \textit{(top right)}. Activity is restricted within the one cell. Dashed lines on traces indicate the time at which the change in \Delta F/F in the first ROI is in excess of 10%. Scale bar: 10\,\mu m
Figure 3.5. The Na\(^+\) channel blocker TTX does not reduce the frequency of spontaneous [Ca\(^{2+}\)]\(_e\) events. Graph shows the mean percentage ± SEM, of cells demonstrating spontaneous activity within a period of 500s in either control solution, or in the presence of TTX (10\(\mu\)M, N=3, P=0.52) in E6 embryos.
Figure 3.6. Spontaneous $\left[Ca^{2+}\right]_i$ activity results from endogenous activation of neurotransmitter receptors. Graphs showing the mean percentage ± SEM of cells demonstrating spontaneous activity within a period of 500s in either control solution, or in the presence of 25µM (A) pirenzipine (N=5, n=500 mitotic and n=500 interphase cells), (B) suramin (N=5, n=250 mitotic and n=250 interphase cells), (C) NBQX (N=6, n=407 mitotic and n=613 interphase cells) or (D) bicuculline (N=6, n=182 mitotic and n=289 interphase cells). *P<0.05. All retinae were E6, except with suramin (see text), where E4 retinae were used.
Figure 3.7. ChAT immunoreactivity is present in the early embryonic retina. A, Cross-section through an E6 retina labelled with anti-ChAT (red), an antibody for choline-acetyltransferase (ChAT). ChAT labels cells and processes throughout the thickness of the retina, including the VZ and immature GCL. B, The same section of retina labelled with Hoechst 33342 (blue). C, Light-microscope image of the same section of retina. D, Negative controls consisted of retinas processed in the absence of primary antibody. Figure shows an example of a negative control stained section from the same retina as shown in A-C. Scale bar 10μm.
Figure 3.8. Spontaneous [Ca^{2+}] activity can be increased by preventing the breakdown of endogenous ACh. A, Co-application of iso-OMPA and eserine (both 100μM) increases the percentage of cells within a random sample showing spontaneous [Ca^{2+}] transients (N=7, P<0.01) in a 500s period. B, Frequency distribution of the number of spontaneous events/cell in control and iso-OMPA/ESerine solution (N=7, E6 embryos). Each event bin is expressed as a percentage of the responses arising within either the control or test group.
Figure 3.9 Spontaneous [Ca$^{2+}$]$_i$ activity can be decreased by increasing the degradation of endogenous ATP. Application of apyrase (80 units/ml; N=3, n=170 \( P<0.01 \)) decreases the number of cells showing spontaneous [Ca$^{2+}$]$_i$ transients compared with controls (N=3, n=175) in E4 retinae.
Figure 3.10. VZ cells in early embryonic retina respond to muscarinic stimulation. 
A, Exogenous application of ACh (50μM) produces oscillatory increases in [Ca^{2+}]_i. 
B, CCh (50μM) mimics the effects of ACh. C, Dose-response curve for CCh in the VZ of E6 retina. The percentage of cells responding at each concentration were 
0μM: 0±0% (N=4); 0.5μM: 1±0.2% (N=4); 1μM: 6±2% (N=4); 5μM: 16±2% (N=3); 
10μM: 48±6% (N=5); 50μM: 88±4% (N=9); 100μM: 96±3% (N=5).
Figure 3.11. The response to CCh is mediated by muscarinic receptors. Muscarine (100 µM, N=4) produces responses in the same proportion of cells as CCh (100 µM, N=7) (87±10% and 93±8%, respectively), while nicotine (100 µM, N=6), stimulates only a small number (6±2%, **P<0.01 compared with CCh). Values plotted are the mean percentage ± SEM. All retinae were E6.
Figure 3.12. The response to CCh is blocked by atropine and pirenzipine. A, The application of 50µM CCh evokes an increase in fluorescence in a typical VZ cell in the E6 chick retina, which can be blocked by co-application of 10µM atropine. The response to CCh returns on washing with Krebs' solution for 10mins. B, The response to CCh is blocked by co-application of the M₁ selective antagonist, pirenzipine (25µM).
Figure 3.13. Pharmacology of the cholinergic response. The responses to both ACh (50µM; light grey) and CCh (50µM; dark grey) are blocked by the broad-spectrum muscarinic antagonist atropine (10µM; N=3 and N=4, respectively) and the M₁-selective antagonist pirenzipine (25µM; N=3 and N=4). The M₂ selective antagonist AFDX 116 (100µM) significantly reduced the response to CCh (ACh was not tested) but did not abolish it (N=7). The M₂-selective antagonist, tropicamide (25µM) blocked the response to CCh (N=4). The effects of antagonists shown as hashed bars. Values plotted are the mean percentage ± SEM; ** P<0.01. All retinae were E6.
Figure S.14. VZ cells in early embryonic retina respond to purinergic stimulation. A, Exogenous application of UTP (50μM) produces oscillatory increases in [Ca^{2+}], which are blocked by co-application of suramin (25μM). The inhibition is reversible. B, Dose-response curve for UTP in the VZ of E4 retina (see text). The percentage of cells responding at each concentration were 0μM: 0±0%; 0.25μM: 14±5% (N=3); 0.5μM: 44±4% (N=3); 1μM: 53±10% (N=3); 2.5μM: 59±2% (N=3); 5μM: 80±9% (N=6); 10μM: 93±4% (N=3); 50μM: 95±2% (N=6); 100μM: 95±2% (N=4).
Figure 3.15. Pharmacology of the purinergic response: agonists. The receptors mediating the E4 purinergic response are strongly activated by UTP (50μM, N=7) and ADP (50μM, N=5). Fewer cells respond to ATP (50μM, N=7) and UDP (50μM, N=4). Values plotted are the mean percentage ± SEM.
Figure 3.16. Pharmacology of the purinergic response: antagonists. The response to UTP (50μM) in E4 retinae is blocked by A, suramin (25μM, N=6), B, Reactive Blue (50μM, N=3) and C, PPADS (30μM, N=3). Values plotted are the mean percentage ± SEM; ** P<0.01.
Figure 3.17. VZ cells in early embryonic retina respond to GABAergic and glutamatergic stimulation. Traces show changes in ΔF/F in single cells. A, Exogenous application of GABA (100μM) produces transient increases in [Ca²⁺], which decline monotonically with time. These are blocked by co-application of the GABA_α antagonist, bicuculline (25μM). The response returns following washout with Krebs’ solution. B, application of glutamate (100μM) also produces responses similar to GABA that are blocked by the AMPA/kainate receptor antagonist NBQX (25μM). Recovery following washout of NBQX was either absent or partial. C, AMPA (100μM) evokes a response similar to glutamate and this response is blocked by NBQX. D, the response to glutamate (100μM) is not blocked by the NMDA receptor antagonist AP5 (up to 50μM). All retinas were E6.
Figure 3.18. The muscarinic and purinergic responses are mediated by the release of Ca\(^{2+}\) from intracellular stores whilst GABAergic and glutamatergic responses result from Ca\(^{2+}\) entry through VOCCs. A, Ni\(^{2+}\) (100µM) greatly reduces the effects of GABAergic (N=3) and glutamatergic (N=3) stimulation but is without effect on responses to either UTP (N=3) or CCh (N=3). B, 10mM caffeine greatly reduces the fraction of cells that respond to either muscarinic (N=6) or purinergic (N=3) stimulation, but is without effect on responses to either GABA (N=3) or glutamate (N=3). Values plotted are the mean percentage ± SEM compared with normalised controls; **P<0.01. All retinas were E6, except with suramin (see text), where E4 retinas were used.
Figure 3.19. The mitotic and interphase VZ cell populations respond to different agonists. At E6, the majority of cells respond to CCh and smaller numbers to UTP, GABA and glutamate. Responses to UTP (100μM, N=4) and CCh (100μM, N=7) arise equally from the mitotic and the interphase populations while those to GABA (100μM, N=4) and glutamate (100μM, N=4) arise predominantly from interphase cells. Insert, a much larger proportion of cells respond to UTP (100μM, N=12) at E4. The response comes equally from the mitotic and the interphase populations. Values plotted are the mean ± SEM.
Figure 3.20. The responses to GABA arise largely from NDCs. A-C, Single confocal sections through the VZ of the same region within an E6 chick retina. A, Hoechst 33342 staining to show the mitotic status of cells (cells indicated by boxes 1 and 2 are in interphase that in box 3 is mitotic). B, A Fluo-4 image taken during the application of GABA (100μM). C, Identification of differentiating neurons using TuJ-1, conjugated with Cy5 (red), and Hoechst 33342 (bright blue). The same three cells are highlighted throughout. Scale bar: 5μm. D, The change in $[\text{Ca}^{2+}]_i$ ($\Delta F/F$) in cells 1-3 in response to GABA. Cell 1 is in interphase, TuJ-1$^+$ and responds to GABA. Cell 2 is in interphase, TuJ-1$^-$ and does not respond to GABA and cell 3 is mitotic, TuJ-1$^-$ and does not respond to GABA. E, Histogram showing the number of TuJ-1$^+$ cells responding to GABA.
Figure 3.21. Developmental changes in sensitivity to neurotransmitter stimulation. 
A. The percentage of mitotic (dark grey) and interphase (stripes) cells in the VZ responding to CCh (N=11), UTP (N=12), GABA (N=8) and glutamate (N=6) (all 100μM) in E4 chick retina. B. The percentage of cells responding to the same agonists at E5 (N=5, N=4, N=6 and N=4, respectively). C. The percentage of cells responding to the same agonists at E6 (N=7, N=4, N=6, N=6, respectively). Values are the mean percentage ± SEM.
Figure 3.22. Cells in the VZ can respond to more than one neurotransmitter. Graphs show the percentage of mitotic and interphase cells responding to more than one of the agonists UTP, CCh, GABA and glutamate (all 100μM) A, at E4 (N=4, n=365) and B, at E6 (N=6, n=385). At E4, virtually all cells respond to both muscarinic and purinergic stimulation. At E6 a significant proportion of the interphase population respond to both GABA and CCh, or to glutamate and CCh. A much smaller proportion of cells were able to respond to both GABA and glutamate. UTP evoked responses in 8% of cells at E6 and these all additionally responded to CCh. None of the cells responding to UTP demonstrated responses to glutamate or GABA at either E4 or E6 (data not shown).
Figure 3.23. The responses to UTP and CCh differ in amplitude and frequency. A, Traces showing examples of the % change in fluorescence ($\Delta F/F$) following the application of 1μM UTP (N=4, n=204, left) or 10μM CCh (N=4, n=73, right). The size of response was larger following the application of 1μM UTP than 10μM CCh. Results are mean± SEM. B, Histograms showing the number of oscillatory peaks in a response following the application of 1μM UTP (left) or 10μM CCh (N numbers as before). Results are expressed as % of total number of cells tested.
Table 1

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Table 2

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GABAergic response

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Figure 3.24. Table 1 shows the potency of muscarinic (pirenzipine), purinergic (suramin), glutamatergic (NBQX) and GABAergic (bicuculline) antagonists (all 25μM) and the Na<sup>+</sup>-channel blocker, TTX (10μM) on the inhibition of spontaneous activity occurring at 36°C in E6 (with the exception of purinergic drugs which were examined at E4—see text) retinae. +++ indicates a significant inhibition. Table 2 shows a summary of the effects of different agonists and antagonists in evoking changes in [Ca<sup>2+</sup>], in the E6 or E4 retina (as above). For agonists, +++ indicates a strong stimulatory effect. For antagonists, +++ indicates a strong inhibitory action. All effects are measured in terms of the ability to stimulate or inhibit a rise in [Ca<sup>2+</sup>].

146a
Chapter 4

The effects of neurotransmitters on mitotic cells in the VZ of the developing chick retina

4.1 Introduction

The production of new neurons and glia by CNS PCs is influenced by many factors. Recent work has centred on the nature and source of these signals in vivo, and how and when they act on the cell cycle, cell survival, and cell growth in order to determine organ size and shape (for review, see Marquardt and Gruss, 2002). There is growing evidence for the involvement of neurotransmitters in the regulation of cell proliferation. ACh, acting via mAChRs, can stimulate cortical PC proliferation (Ma et al., 2000; Li et al., 2001), and purine nucleotides and nucleosides can increase or decrease DNA synthesis in glia and neurons (Ciccarelli et al., 1994, Sugioka et al., 1999). GABA has also been reported to be able to both increase (Fiszman et al., 1999; Haydar et al., 2000) and decrease (LoTurco et al., 1995) cell proliferation, and to partially block the mitogenic actions of bFGF in the cortex (Antonopoulos et al., 1997). Like GABA, glutamate may also increase or decrease cell proliferation in the cortex by changing the cell cycle time; both glutamate and GABA increase the size of cortical VZ clones, while decreasing SVZ clone size (Haydar et al., 2000). In the cortex, LoTurco et al. (1995) have shown that, when applied alone, GABA_A and AMPA/kainate receptor antagonists increase DNA synthesis, while when applied together they decrease it.

The use of time-lapse imaging of cortical slices has suggested that the cleavage plane of PCs may predict the fate of daughter cells. In a symmetrical division two identical daughter cells are produced that then re-enter the proliferative cycle. Asymmetric division gives rise to one daughter cell that behaves like a young migratory neuron and another that continues in the proliferative cell cycle. In the cortex, an increase in the rate of differentiation is correlated with an increase in the proportion of asymmetric divisions (Caviness et al., 1995; Takahashi et al., 1994, 1995). There is extensive variation in the orientation of division in mitotic cells in the developing nervous system. A recent hypothesis proposes that the orientation, with respect to the plane of the tissue,
of the cleavage plane during mitosis determines whether a division is symmetric or asymmetric. Thus, if the cleavage plane lies perpendicular to the plane of the cortex (a 'vertical' division) the division is thought to be symmetric, whilst when the cleavage plane is parallel to the ventricular surface (a 'horizontal' division), the resulting division is asymmetric (Chenn and McConnell, 1995).

The RPE plays an important role in the normal development of the neural retina, although the nature of the signals between the RPE and the neural retina are unclear. ATP is released from adult human RPE cells in a polarised manner, into the sub retinal space (Mitchell, 2001), and can cause a rise in $[\text{Ca}^{2+}]_i$ in cells in the immediately adjacent neural retina (see Chapter 3 and Pearson et al., 2002; Sakaki et al., 1996). The function of ATP release during early development has received little attention.

This chapter describes the role of muscarinic, purinergic, glutamatergic and GABAergic receptors in the regulation of the cell cycle in the embryonic chick retina. Experiments were carried out to determine the effects of these neurotransmitters on the rate of mitosis in retinal whole-mounts, and on eye growth and mitosis in the retina when applied in ovo. The final part in the chapter describes experiments that examine a potential role of the RPE in regulating proliferation in the neural retina.
4.2 Methods

The methods used in this chapter are described in detail in Chapter 2, sections 2.5 and 2.6

4.2.1 Real-time imaging of mitosis and metaphase spindle rotation

E5 retinae were dissected and loaded with 2μM Hoechst 33342. Where possible, one eye from each embryo was perfused with drug-containing Krebs’ solution and the other served as the control. Retinae were mounted with the VZ facing the objective of an inverted confocal microscope, except in experiments examining the influence of the RPE where preparations were mounted with the GCL facing the objective, and were imaged at 36°C. xy images of the Hoechst-stained chromatin in the VZ were acquired at 15s intervals for 90mins. Individual mitotic cells were selected at random for analysis and the time from which condensed chromatin is first seen as a rod-like structure to the first separation of the chromosomes (i.e. the length of time spent in metaphase) was measured. These data were averaged and compared with the time it took cells to progress through metaphase in the corresponding region in the control eyes.

Experiments investigating the effects of the RPE on neural retinal mitosis were performed as above, although the RPE was left intact in the appropriate experiments.

To eliminate variations between animals these experiments were done using one eye as a control and the opposite eye to study the effects of drugs. In order to eliminate the effects of time following dissection of eyes, the order of use of eyes as either control or experimental were varied. It was not always possible to use paired retinae due to movements during the imaging process or, where the RPE was used, loss of the RPE’s attachment to the neural retina. However, in the event the differences in the times spent in metaphase were small from one eye to another and data were pooled for each experimental group, and for the respective controls; it may be assumed that paired assessment would act to increase the statistical significance of the findings shown here.

When viewed as though looking down on the VZ, metaphase plates with a rod-like appearance constantly rotate around a central axis, the rotations ceasing upon entering.
anaphase. In section 4.3.2, quantitative analysis of the rotational movements made by the metaphase mitotic apparatus was done on a sample of cells imaged for the experiments described above. The orientations of the mitotic apparatus during metaphase were taken as the angle of a line drawn along the length of the bar of stained chromatin (metaphase rod) of a dividing cell. The orientation at anaphase was determined by drawing a line parallel to the two parting clusters of chromatin (see Figure 2.3, Chapter 2). Measurements were taken from every 5<sup>th</sup> image in a movie. The angle of a metaphase rod at <i>n</i> time points was measured relative to the final angle adopted by the chromatin of that cell at anaphase. For each cell, the average of all measurements taken during anaphase was calculated and each measurement taken during metaphase and anaphase was normalized relative to the mean anaphase angle (0°). Thus, 0° corresponds to an orientation that is parallel, and 180° corresponds to an orientation that is anti-parallel, to the mean angle at anaphase. Time sequences are presented as polar plots of orientations versus time. The distribution of orientations during metaphase, relative to the angle adopted at anaphase, was assessed in a population of 20 cells. Measurements were sorted into those occurring during metaphase, and those occurring after the onset of anaphase. These measurements were then sorted into 10° bins and plotted as rose diagrams, where the area of each sector is proportional to the number of observations occurring at a given angle.

4.2.2 Application of drugs in ovo

Sufficient eggs for each neurotransmitter group (including agonist, antagonist and control) were set at the same time and “windowed” at E5 and injected with agonists (final concentration in the egg ~50μM), antagonists (final concentration ~25μM) or PBS. Injections were made into the amniotic pouch. Whilst full staging was not possible prior to drug-application, the windowed shells enabled a visual assessment of all embryos, in ovo, and ensured that they were at a similar stage in development. Eggs were resealed with Sellotape and incubated for 8h at 36°C. The embryos were then fixed with 4% PFA. The eye diameter was measured along a line through the choroid fissure and the body length was measured from the base of the head to the tail tip. The drug-treated groups were tested against the respective control group. The diameters of
eyes treated with drugs are expressed as a fraction of the mean of the respective control group.

Transverse frozen sections were taken and those passing through the centre of the retina were labelled with Hoechst 33342 (2μM). Three non-adjacent sections from each eye were taken and 10 regions/section were selected at random and imaged. Mitotic cells were identified from their condensed chromatin and all stages from prophase to telophase were counted. The number of mitotic cells/100μm length of retina was calculated. Final results are shown as the percentage difference compared to the mean of the respective controls. The angle of division, with respect to the RPE, was measured for those profiles clearly in metaphase (see section 2.6.2 and Figure 2.4 for full details). A division in which the metaphase rod was aligned at right angles to the plane of the RPE corresponds to an angle of division of 90° (a ‘vertical’ division, see Chapter 2) and one in which the metaphase rod lay parallel to the plane of the RPE corresponds to an angle of division of 0° (a ‘horizontal’ division, see Chapter 2). Cell density counts were carried out on each eye by randomly selecting three or more 5000μm² regions from at least three sections immediately adjacent to the optic nerve for each embryo. The number of Hoechst-stained nuclei within each area was counted and an average determined for each drug-treated embryo before being compared against controls.

4.2.3 Statistical analysis

In section 4.3.2, data is presented that describes rotations of the metaphase rod around a central axis. The mean vector (μ), vector length (r) and Rayleigh’s coefficient were calculated for the distribution of angles at metaphase and at anaphase. The data were accurately described by a Von Mises distribution and so the Watson’s F-test for two circular means was used to determine whether or not the mean vector lengths of the two populations were significantly different.

Data presented in sections 4.3.3-4.3.8 were normally distributed, as assessed using the Kolmogorov-Smirnov test (GraphPad Prism 3.00) and the following statistical tests were applied: i) sections 4.3.3-4.3.6 and 4.3.10 involve measurements of the time spent in metaphase. Each agonist or antagonist group was tested against its own respective
control group consisting of data from the contralateral eyes using an unpaired Student's t-test, ii) in sections 4.3.7 and 4.3.8, data from embryos treated with the respective agonists and antagonists for a given neurotransmitter receptor were compared against the same control group, set to develop at the same time as the test animals. Statistical significance was determined using ANOVA and Dunnett’s test for multiple comparisons against a single control group.

Data concerning the distribution of angles of division between vertical and horizontal orientations (section 4.3.9) fitted a non-normal distribution. The Kruskal Wallis rank test was applied to determine the effect of agonists and antagonists on the distributions of angles. ‘Vertical’ and ‘horizontal’ divisions were defined as cells dividing at the arbitrary angle of ≥45° and <45° respectively. Statistical differences in the proportions of cells dividing vertically or horizontally were assessed using the Mann Whitney-U test.

Differences were considered statistically significant at one of two levels: *P<0.05 and **P<0.01. The results are means ± SEM of each control or test group, where N=number of retinae investigated and n=number of cells recorded.
4.3 Results

I. The effect of neurotransmitters on mitosis in the neural retina

4.3.1 Mitosis can be followed in real time in whole mount retinae

Many of the experiments implicating neurotransmitters in the control of cell proliferation have been carried out in vitro and do not distinguish effects on mitosis from those which affect other aspects of the cell cycle. Here, Hoechst 33342 labelling of chromatin has been used to directly image the effects of purinergic, muscarinic, glutamatergic and GABAergic stimulation and blockade on mitosis in the retinal VZ.

Cells in the VZ of E5 retinae were imaged using a x63 lens with x0.7 zoom. A typical field of view (18x10^3 μm^2) contained 490±5 cells (N=18) and included cells in all stages of mitosis and in interphase (Figure 4.1b). In control preparations, more than 100 cells (102±10 cells, N=8) could usually be followed from prophase to cytokinesis during the 90min recording period. PCs with uncondensed nuclear material typical of cells in interphase could be observed arriving at the VZ. Upon entry into the VZ, condensation of chromatin followed with a delay of between 15s and 60mins. Once mitosis was initiated the time cells spent in prophase was quite short (4±3mins N=8). The transition between prophase and metaphase was abrupt. Only 2±0.5 cells (N=8, n=4085) in a field of view were in prophase at any one time. In metaphase, the chromatin pairs line up along the mitotic equator. The majority of divisions at this time are ‘vertically’ orientated with respect to the VZ (Chenn and McConnell, 1995), such that the mitotic equator and cleavage plane lie perpendicular to the plane of the VZ (Figure 4.1a). When viewed as though looking down on the VZ the chromatin appears as a rod, located in the centre of the cell. 3D reconstructions of 0.5μm optical sections through the VZ show that vertically aligned metaphase “rods” are disk-shaped when viewed from the side (Figure 4.1c, top). Cells in anaphase are typified by having two smaller, parallel bars of chromatin that become rounded as they enter telophase. In contrast, interphase cells appear small and round when viewed in xy, xz or yz cross-sections (Figure 4.1c, bottom). Approximately 10-20% of divisions at this time are ‘horizontal’ (see section 4.3.9 and Chenn and McConnell, 1995), such that the mitotic equator, and cleavage
plane, lies parallel with the VZ, and the metaphase plate appears rod-like from the side (Figure 4.1a). Small numbers of cells with this appearance were observed; these progressed to cytokinesis and the daughter cells then migrated from the VZ. The behaviour of these cells could be followed using the z-line function of the confocal microscope. However, z-line scans typically incorporated too few horizontally dividing cells to enable systematic study and slight movements in the xy plane made their behaviour difficult to follow. For these reasons, horizontal divisions were not studied in detail.

The period between entry into metaphase and chromosome separation was timed for vertically dividing cells. Figure 4.2 shows an example of one cell for which this was done. It should be noted that individual chromosomes could not always be resolved and so it was not possible to distinguish pro-metaphase from metaphase, and thus here the whole period is referred to simply as metaphase. In each field of view the time spent in metaphase was measured for up to 50 randomly selected cells that entered prophase during the 90min imaging period. Since the average time spent in metaphase in control retinae was ~30mins (see below), a period of 90mins was sufficiently long to encompass all of the later stages of mitosis for the vast majority of cell divisions. Following anaphase, the nuclear material of the daughter cells became rounded as it de-condensed during telophase. After completion of cytokinesis the two cells migrated from the VZ and out of the focal plane. Cells could also be seen entering the VZ and to begin mitosis. Thus, despite UV illumination and the absence of the RPE, PCs divided and the progeny migrated both from and into the VZ.

4.3.2 Time-lapse imaging of mitosis: the rotations of metaphase plates within the plane of the VZ

The process of mitosis is highly dynamic. Early in metaphase the chromatin of dividing cells presented either a rod- or a disk-like appearance (see Figure 4.1c), indicating that the alignment of the spindle parallel to, or perpendicular with, the plane of the VZ occurs rapidly. Once aligned, cells rarely changed their orientation, although very occasionally the rod-like appearance of a metaphase plate transformed into a disk, an occurrence that suggests that reorientations of the plane of division are possible, if infrequent (Figure 4.3a). However, in most instances the plate rapidly returned to lie
perpendicular with the VZ. When viewed as though looking down on the VZ, metaphase rods often rotated within cells (Figure 4.3b). The extent of these movements ranged from a few degrees to large excursions encompassing 360° or more. There were also many changes in the direction of movement (clockwise or anti-clockwise). The total angular displacement could include one or more complete rotations, indicating that the entire mitotic apparatus, including the spindle, rotates rather than a simple realignment of the chromatin within the spindle complex. Movement was continuous during metaphase but stopped abruptly once cells entered anaphase. The chromosomes pulled apart and the cells proceeded through cytokinesis with almost no change in orientation of the mitotic apparatus relative to the tissue. After completion of anaphase, some daughter cells immediately migrated out of the plane of focus and into the neural retina, while others remained at the apical surface for several minutes.

Thus, rod-like metaphase plates rotate constantly and markedly about a central axis within the plane of the VZ throughout metaphase, the movement ceasing upon entering anaphase. These movements were plotted as polar graphs for each of a sample of cells, some of which are shown in Figure 4.4. In these, the angle of orientation of the metaphase rod is shown relative to that at anaphase (0°). The distance of the data point from the centre point reflects time and its angular position, the angle of the metaphase rod. The changes in the orientation of the metaphase rods were re-plotted as "rose diagrams" (see Figure 4.4) in which the area of each 30° bin is proportional to the frequency of occurrence of the angles within the bin. In many cells, the metaphase rod spends most of its time at an angle near that at which it later enters anaphase and divides (e.g. Figure 4.4a-c). The large majority of cells demonstrated this behaviour. However, a smaller population of cells showed more extensive movements, with a less clear preference for a particular orientation (e.g. Figure 4.4d-e).

The changes in the orientation of the metaphase rods during metaphase and anaphase were calculated for a population of 20 cells (N=4). For each cell the measurements of rod orientations were separated into those occurring during metaphase and those occurring after the onset of anaphase, before being pooled with those from the other cells. These data were counted into 10° bins and the distribution plotted as a rose diagram. In metaphase, the distribution of metaphase rod orientations was broad;
metaphase rods were seen in all orientations between 0° and 360°. In contrast, the
distribution of orientations measured after the onset of anaphase showed very little
change once the separation of chromosomes had begun (Figure 4.5). However, as
Figure 4.5 shows, the distribution of angles observed during metaphase are also
concentrated in the quadrant 0±45°. 62% of measurements occurred within 0±45°
compared with 22% and 13% in the quadrants 90±45° and 270±45°, respectively. This
suggests that whilst cells in metaphase rotate continuously, they show a bias toward the
angle later adopted in anaphase. This distribution of angles fits a Von Mises, unimodal
model of circular distribution (P<0.0001). The mean vector (\(\mu\)) and the length of mean
vector (\(r\)) of the metaphase and anaphase data sets were calculated. The mean vector, \(\mu\),
describes the mean angle of the data set, whilst \(r\) (a value between 0 and 1) indicates the
strength of the mean angle relative to all other angles. At anaphase \(\mu=359±1.0°\) and
\(r=0.99\) (n=97 observations) indicating that the mean angle is very close to 0° and that
almost all the data falls close to this value. The mean vector at metaphase is very close
to that at anaphase (\(\mu=8.3±3.2°\), \(r=0.54\), n=463 observations). The Rayleigh coefficient
gives a statistical measure of whether the mean vectors have significant magnitudes, and
gives an indication of the uniformity of the distributions. It is defined as:

\[
\text{Rayleigh's coefficient} = 2n(r^2)
\]

where \(n\) = total number of observations and \(r\) = vector length of the total data set.

The magnitude of the mean vectors of both the metaphase and anaphase data sets were
very significant (metaphase, Rayleigh's coefficient=273.8, P<0.0001; anaphase,
Rayleigh's coefficient=188.3, P<0.0001) showing that both distributions have a strong
bias towards the mean vector. Calculation of the Watson F statistic shows that the mean
angles at metaphase and anaphase are not significantly different (F=3.56, P=0.11).
Together, these tests show that during metaphase, rods can undergo large rotational
excursions but show a strong preference toward the angle that is later adopted during
anaphase.

Purinergic and muscarinic stimulation have profound effects on the speed of mitosis and
the level of proliferation in the embryonic chick retina (see section 4.3.3). In order to
examine whether or not purinergic or muscarinic receptor activation had any effect on
the extent of rotational movement during metaphase, UTP (10µM) or CCh (10µM) were applied to whole-mount E5 retinae throughout the imaging period. A random sample of cells undergoing mitosis was analysed for i) the total rotational movement each cell underwent during metaphase, and ii) the average rotational movement/min. Control cells spent 33±2mins\(^1\) in metaphase and underwent an average of 495±39° (N=8, n=40) of rotational movement during this time. Cells rotated an average of 13±1°/min (N=8, n=40). Purinergic stimulation reduced the length of time spent in metaphase (15±2mins, N=4, n=20) and concurrently decreased the total rotational movement from 481±24° (N=4, n=20), seen in the matched control eyes, to 127±30° (N=4, n=20, paired Student’s t-test P<0.01) in the presence of UTP. Conversely, muscarinic stimulation greatly increases the length of time spent in metaphase (57±2mins, N=4, n=20; see section 4.3.3) and the total rotational movement compared to the corresponding controls (1178±180°, N=4, n=20, P=0.01 and 509±55°, N=4, n=20 respectively). Expressing the extent of rotation as a function of time shows whether or not cells rotate at a slower or faster rate in the presence of drugs, compared with controls. Purinergic stimulation had no discernible effect; cells rotated 10±1°/min (N=4, n=20, P=0.43) and 11±1°/min (N=4, n=20) in the presence of UTP and control solutions, respectively. Cells appeared to rotate at a slightly faster rate in the presence of CCh, although the results did not reach statistical significance (P=0.06). Controls rotated 14±2°/min (N=4, n=20), whilst those exposed to CCh rotated an average of 29±6°/min (N=4, n=20).

4.3.3 Muscarinic and purinergic, but not GABAergic or glutamatergic, receptors affect mitosis

This section describes a series of experiments that investigate the effects of muscarinic, purinergic, GABAergic and glutamatergic receptors on the speed of mitosis in the E5 chick retina. When retinae were exposed to UTP (10µM) the average length of time spent in metaphase was significantly reduced from 32±5mins in control preparations, to only 12±2mins (N=6, n=99, unpaired Student’s t-test P<0.01) (Figure 4.6). This effect was absent when UTP was applied to E7-8 retina (control cells spent 30±1mins in metaphase compared with 29±2mins in the presence of UTP, P=0.55, unpaired t-test,\(^1\) The data sets presented in this section are from a random sample of recordings taken from the movies of mitosis described below. See section 4.3.3 for further details.

157
N=3, n=150 for both). In contrast, the muscarinic agonist, CCh (10μM) significantly extended this interval from 33±5mins in E5 controls to 62±6mins (N=4, n=117, P<0.01). Neither glutamate, nor GABA (both 20μM), had any significant effect (N=5, n=105, P=0.55 and N=5, n=82, P=0.86, respectively); the time spent in metaphase being 35±5 and 33±5mins, respectively, compared with appropriate control times of 34±4 (N=3, n=180) and 32±3mins (N=4, n=110), respectively. Thus, purinergic agonists reduced the length of time spent in mitosis whilst muscarinic agonists extended it (Figure 4.6).

Given the effects of muscarinic and purinergic stimulation, similar experiments were done using pirenzipine or suramin (both 25μM) to establish whether or not blocking endogenous muscarinic and purinergic signalling had any effect on mitosis (Figure 4.7). Pirenzipine significantly reduced the time spent in metaphase, compared to controls (11±0.3mins versus 32±1mins in controls; N=3, n=86, P<0.01). Thus, muscarinic antagonists induce a change opposite to that caused by muscarinic agonists. Suramin increased the time spent in metaphase but this was not significant (37±1mins versus 31±1mins in controls; N=4, n=63, P=0.56).

4.3.4 Caffeine increases the time spent in prophase

Purinergic and muscarinic [Ca^{2+}]_i responses arise from the release of Ca^{2+} from intracellular stores and are blocked by exposure to caffeine (10mM) (see Chapter 3). E5 retinae were pre-incubated for 10mins, and then perfused with, caffeine (10mM) to investigate whether emptying Ca^{2+} stores had any effect on mitosis. The application of caffeine prevented cells from progressing beyond prophase. In control solution, the same proportion of cells (although not the same cells) was in prophase at the end of the experiment as at the outset (2±0.5 cells versus 2±1 cells/18x10^3μm^2; N=4, n=1919 ). During the 90min period of the experiments, many cells (103±10 cells/18x10^3μm^2, N=4) in control retinae progress through all stages of mitosis and eventually divide. However, in retinae treated with caffeine, the number of cells in prophase at the start of recording (following the 10min incubation period) was raised to 19±14 cells/18x10^3μm^2 and this increased to 119±19 cells (N=3, n=1426, P<0.01) over the 90 min recording.

\[18x10^3\mu m^2\] is the area of a field of view at x63 magnification with x0.7 zoom
period. Many cells in caffeine-treated retinas that entered the VZ during the period of observation underwent chromosome condensation but failed to progress further. In 3 preparations treated with caffeine only 5 cells passed through all stages of mitosis during the recording period. The time spent in metaphase by the 5 cells ranged from 23 to 78 mins. The majority of cells in metaphase at the start of the recording period did not progress into anaphase in the presence of caffeine (data not shown). By contrast, all cells in control retinas that were in metaphase at the outset of recording progressed into anaphase and on to cytokinesis.

4.3.5 BAPTA-AM blocks the purinergic and muscarinic $[Ca^{2+}]_i$ responses, reduces spontaneous activity and prevents entry into metaphase

The $Ca^{2+}$-chelator BAPTA-AM prevents the changes in $[Ca^{2+}]_i$ induced by UTP and CCh, and greatly reduces the level of spontaneous $[Ca^{2+}]_i$ transients. Pre-incubation of E5 retinas with 100$\mu$M BAPTA-AM reduced the proportion of cells responding to CCh from 85% in controls to $6\pm3\%$ (N=3, n=160, P<0.01, unpaired Student’s t-test). Similarly, the response to UTP was reduced from 81% to $8\pm5\%$ (N=3, n=160, P<0.01). The number of spontaneous $[Ca^{2+}]_i$ events occurring in the VZ was reduced by $80\pm7\%$ in the presence of the chelator. Spontaneous events were seen in $29\pm3\%$ of control cells (N=3, n=150) compared to $6\pm2\%$ (N=3, n=128, P<0.01) in BAPTA-AM treated retinas.

As with caffeine, pre-incubation of retinas with BAPTA-AM prevented cells from progressing through mitosis. In control preparations large numbers of cells ($100\pm9$ cells/18x10$^3$$\mu$m$^2$, N=4) could be followed from prophase through to anaphase over the 90 mins period of recording. The proportion of cells in prophase at the outset ($2\pm0.5$ cells/18x10$^3$$\mu$m$^2$, N=4, n=2166) was the same as at the end of the recording period ($3\pm0.5$ cells). However, in retinas incubated with BAPTA-AM for 30 mins prior to recording, $41\pm8$ cells/18x10$^3$$\mu$m$^2$ (N=6, n=2444, P<0.01) cells were in prophase at the start of recording and virtually all remained in this state for the 90 min duration of the experiment. A total of $156\pm23$ cells/18x10$^3$$\mu$m$^2$ were in prophase at the end of the recording period. $18\pm8$ cells/18x10$^3$$\mu$m$^2$ in retinas treated with BAPTA-AM progressed from prophase through to anaphase, compared with $100\pm9$ cells/18x10$^3$$\mu$m$^2$ in controls.
The cells that did pass through all stages of mitosis were in metaphase for 26±3mins (n=117, N=6).

4.3.6 Purinergic, but not muscarinic, stimulation partially rescues cells from the effects of caffeine

The agonists UTP or CCh were co-applied with caffeine to investigate whether they influence the rate of mitosis after the depletion of intracellular Ca\(^{2+}\) stores. Retinae were pre-incubated with 10mM caffeine for 10mins. Recordings (90mins) were then carried out in the continued presence of caffeine and either UTP (10\(\mu\)M) or CCh (50\(\mu\)M).

Co-application of CCh and caffeine produced similar results to those with caffeine alone. 88±4 cells/18x10\(^3\)\(\mu\)m\(^2\) (N=4, n=1805) were in prophase at the end of the recording period. By comparison, in controls similar small numbers of cells were found in prophase at the outset and end of the recording period (2±0.5 cells versus 1±1 cell; N=4, n=1830). In all control retinae, 106±11cells/18x10\(^3\)\(\mu\)m\(^2\) (N=3) passed through all stages of mitosis during the recording. The time these cells spent in metaphase was 26±2mins (N=4, n=200). In contrast, only 6±2 cells/18x10\(^3\)\(\mu\)m\(^2\) (N=3) in the retinae exposed to caffeine and CCh progressed through mitosis. These cells took an extended length of time upon entering prophase to progress to anaphase, with the time spent in metaphase increasing to 49±5mins (N=3, n=18, P<0.01).

The presence of UTP appears to ‘rescue’ a proportion of prophase cells from the block caused by caffeine. The number of cells apparently halted in prophase was increased significantly compared with controls (72±13 versus 1±1 cells, N=3 for each, P<0.01). However, whilst only very small numbers of cells (<6cells/18x10\(^3\)\(\mu\)m\(^2\)) could be followed throughout all stages of mitosis in the presence of caffeine alone, or caffeine and CCh, in the presence of UTP and caffeine ~40 cells/18x10\(^3\)\(\mu\)m\(^2\) could be followed from prophase to anaphase during the recording period, compared with 102±6 cells in controls. Of the total number of cells entering prophase during the period tested approximately half continued through to metaphase and then anaphase. In the presence of UTP, mitotic cells in caffeine-treated retinae spent roughly the same time in
metaphase as in control retinae in the absence of caffeine (28±1 mins, N=3, n=150 versus 26±2 mins in controls, N=3, n=150, respectively).

II. The effects of in ovo application of neurotransmitters on retinal development

4.3.7 Muscarinic and purinergic, but not GABAergic or glutamatergic, receptors affect eye growth

Only a short part of the cell cycle, mitosis, can be easily observed by in vivo imaging of chromatin. In order to examine further the potential effects of muscarinic, purinergic, GABAergic and glutamatergic receptor stimulation and blockade on the cell cycle, other experiments were conducted. Firstly, the effects on eye growth of agonists and antagonists of these four neurotransmitter systems were examined. Secondly, investigations were carried out to determine the effects of these agents on the number of mitotic figures seen in the VZ. E5 embryos were allowed to develop, in ovo, for 8h in the presence of CCh, pirenzipine, UTP, suramin, PPADS, Reactive Blue, GABA, bicuculline, AMPA or NBQX (final concentrations in the egg: ~25μM for antagonists and ~50μM for agonists. See section 2.6). The data for each neurotransmitter agonist/antagonist were tested against the same control group using ANOVA and Dunnett's test for multiple comparisons.

Embryos grown in the presence of UTP had eyes 10±2% larger in diameter than controls (N=4, P<0.02 following correction for multiple comparisons) (Figure 4.8), an effect most likely due to an increase in cell number. This result is consistent with the finding described above for the effects of UTP on mitosis, with increased mitotic rate leading to an increased cell number after 8h. Application of suramin in ovo was lethal. However, PPADS and Reactive Blue caused a reduction in eye size of 21±3% (N=4, corrected P<0.01) and 18±1% (N=3, corrected P<0.02), respectively.

In contrast to the effects seen with UTP, and congruent with its effects on mitosis, CCh caused treated eyes to be 8±3% smaller than those in control embryos (N=10, corrected P<0.05) (Figures 4.8 and 4.9). The muscarinic antagonist pirenzipine induced a change opposite to that of CCh, eyes in the treated embryos having a diameter 8±2% greater
than that in controls. These findings are consistent with the reduction in the rate of mitosis produced by muscarinic stimulation leading to a reduction in cell number.

Neither GABA nor bicuculline had any significant effect on eye diameter (Figure 4.8), GABA reducing eye size by 3±2% (N=6, P=0.66) and bicuculline increasing it by 3±3% (N=6, P=0.80). Application of AMPA and NBQX were similarly without significant effect, AMPA reducing eye size by 3±3% (N=6, P=0.90) and NBQX reducing it by 2±2% (N=6, P=0.99).

Drugs applied in ovo can affect any of the tissues within the embryo. For example, rather than affecting eye size directly muscarinic and purinergic agents may increase or decrease heart rate leading to increased or decreased rates of development. However, eye size does appear to be affected by muscarinic and purinergic agents, independent of overall development. Throughout these experiments, control and drug-treated embryos were started at the same time and only those at the same stage of development were used. Measurement of embryonic body length after fixation revealed no significant variations between any drug-treated group and their respective controls (minimum P value for any drug-treated group compared with control group was 0.28).

It has been previously reported that small eye defects may result from a normal-sized neural retina developing within an abnormally small eye capsule (Coulombre, 1956). Histological retinal sections taken from the eyes of the embryos described above were examined in order to ensure this was not the case here. In all cases of small eyes, the neural retina appeared to be reduced in proportion to the rest of the eye, was not folded and was of normal thickness. Measurements of cell density were taken to ensure that the apparent changes in eye diameter were not due to cell swelling or shrinkage. Cell density counts were performed on retinal sections immediately adjacent to the optic nerve. The number of cells in at least three 5000μm² regions in three different sections from each retina was counted and compared against controls (Figure 4.10a-c). There was no significant difference between the different conditions and cell density remained constant (lowest P value was 0.74). In embryos treated with muscarinic drugs the average number of cells/5000μm² was in controls, 101±3; CCh-treated, 100±3 (P=0.73);
and pirenzipine-treated, 99±3 (P=0.65). Values for purinergic-treated embryos were: controls, 95±3; UTP, 94±3 (P=0.76); and PPADS, 94±3 (P=0.73). Similarly, neither GABA nor AMPA, nor the respective antagonists, had any significant effect on cell density (Controls=96±2; GABA=96±2, P=0.98; bicuculline=96±1, P=0.92 and controls=103±2; AMPA=102±1, P=0.69; NBQX=101±1, P=0.37, respectively).

Applied at the concentration and in the manner described above, CCh, pirenzipine, UTP, PPADS, Reactive Blue, GABA, bicuculline, AMPA and NBQX had no significant effect on the number of dying cells in the retinæ when used in ovo. The exception was suramin, which was lethal. Hoechst staining was used to reveal pyknotic nuclei (which appear small, rounded and densely stained) as markers of dying cells. Pyknotic nuclei were absent from the vast majority of sections examined. Figure 4.10d shows the proportion of pyknotic profiles/100μm length of retina as a percentage compared with the respective control embryos. The numbers of pyknotic nuclei were small and did not differ for any drug treatment from that in controls (minimum P value 0.31). These results suggest that there is little or no increase of cell death associated with in ovo application of either PPADS or CCh, and that apoptosis is a rare event at this time, unless the time taken to clear dead cells from the retina is very short.

4.3.8 Muscarinic and purinergic, but not GABAergic or glutamatergic, receptors affect the number of cells in mitosis in the VZ

If a manipulation affects mitosis and proliferation there should be a concomitant change in the number of mitotic cells present in the ventricular region (for further discussion see Rakic, 2002). Retinal sections were taken from the embryos above (see section 4.3.6) that had been treated with CCh, pirenzipine, UTP, suramin, PPADS, Reactive Blue, GABA, bicuculline, AMPA or NBQX, and the number of mitotic profiles/100μm length of the retina determined (Figure 4.11). Retinæ exhibit temporal gradients of development. To test whether cell division patterns were significantly influenced by these gradients at E5, counts were carried out on non-adjacent sections at 3 different locations between the retina centre and periphery. Position was found to have little effect on the number of mitotic cells and so data from all regions of the retina were combined in the final analysis. Embryos exposed to UTP showed a 45±4% increase in
the number of mitotic cells in the VZ (N=4, corrected P<0.01) compared with controls. PPADS and Reactive Blue caused the opposite effect, significantly reducing the number by 54±5% (N=4, corrected P<0.01) and 51±11% (N=3, corrected P<0.01), respectively, compared with controls (N=4).

By contrast, CCh reduced the number of mitotic profiles by 21±4% (N=10, corrected P<0.05) (Figures 4.10a-c and 4.11), while pirenzipine increased the number by 44±11% (N=10, corrected P<0.01). Ectopic divisions were not observed, so a decrease in mitotic profiles in the VZ is unlikely to be due to a failure of PCs to reach the VZ. Neither GABA (N=6, P=0.68), nor bicuculline (N=6, P=0.65), had any significant effect on the number of mitotic profiles in the VZ, both drugs increasing mitotic cells by 6±11% compared with controls. Similarly, no effect was detected on the number of mitotic profiles with either AMPA (increase of 11±5%) or NBQX (decrease by the same amount) (N=6, P=0.66 and N=6, P=0.82, respectively, see Figure 4.11). These results are consistent with the effects of these drugs on both the changes in eye diameter and the rate of mitosis seen in the living retina and strongly suggest a role for muscarinic and purinergic receptors, but not glutamate or GABA receptors, in the regulation of cell number in the developing retina.

4.3.9 The plane of cell division is unaffected by neurotransmitter receptor activation

Several investigations have led to the proposal that the orientation of the mitotic cleavage plane in the cortex determines whether a PC divides symmetrically or asymmetrically (Chenn and McConnell, 1995; Chenn et al., 1998). Vertical divisions, in which the metaphase rod lies perpendicular to the plane of the cortex, have been proposed to be indicative of symmetrical divisions. In contrast, when the cleavage plane is horizontal to the ventricular surface, the resulting division is thought to be asymmetric, producing one PC and one NDC (Figure 4.12a). Similar principles are thought to apply for the retina.

The experiments described below examine the orientation of the cleavage plane in the chick retinal VZ and the effects of neurotransmitter receptor activation or blockade on the proportions of cells dividing vertically (symmetrically) or horizontally (asymmetrically). The retinal sections prepared for the experiments described in 4.3.7
were analysed to determine the orientation of the cleavage plane and hence cell division. This was determined by measuring the angle of the metaphase rod, relative to the RPE, for each cell in metaphase or anaphase (Figure 4.12b, see section 2.6.2 for further details).

Cleavage planes were measured and sorted into $10^\circ$ bins. In control E5 chick retinae, most PC divisions were vertical, with the cleavage-plane aligned roughly perpendicular to the RPE (i.e. the metaphase rod is at right angles to, and the spindle plane is assumed to parallel with, the RPE). However, many cells illustrated cleavage planes at angles between 0 and $90^\circ$, with respect to the plane of the RPE (Figure 4.13). The distributions of the angles of division for embryos exposed to purinergic, muscarinic, GABAergic and glutamatergic agents are given in Figures 4.14, 4.15, 4.16 and 4.17 respectively. Application of purinergic, muscarinic, GABAergic or glutamatergic agonists (all 50μM) or antagonists (all 25μM) had no effect on the distribution of angles at which cells appeared to divide. The Kruskal Wallis statistic (KW) and P values for embryos treated with purinergic and muscarinic, GABAergic or glutamatergic drugs, compared with their controls, were KW=4.17, P=0.12; KW=0.605, P=0.74; KW=0.11, P=0.94 and KW=0.05, P=0.97, respectively.

Cells were also grouped into ‘vertical’ or ‘horizontal’ divisions. An arbitrary angle of $\geq45^\circ$, relative to the tissue plane, was used to classify a division as ‘vertical’ and $<45^\circ$ to classify a division as ‘horizontal’. The proportion of cells that underwent vertical divisions in the control groups ranged between 75 and 88% with a mean of $82\pm3\%$ (N=27). The number of vertical cell divisions occurring in the E5 chick retinal VZ was unaffected by any of the agonists or antagonists tested. Vertical divisions comprised $82\pm3\%$, $88\pm1\%$ and $89\pm1\%$ of divisions in embryos injected with control (PBS) solution (N=8), UTP (N=4; Mann Whitney U statistic (U)=14, P=0.44) and PPADS (N=4; U=8, P=0.10) respectively. Vertical divisions comprised $75\pm3\%$, $77\pm2\%$ and $78\pm2\%$ of divisions in embryos exposed to control solution (N=7), CCh (N=10; U=11, P=0.08) and pirenzipine (N=10; U=14, P=0.37) respectively. Similarly, application of GABA (88±2%; N=6; U=13.5, P=0.53) or bicuculline (88±2%; N=6; U=16, P=0.88),

3 Whilst metaphase rods rotate within the plane of a vertical division (this chapter; Adams, 1996) the angle of a metaphase rod, with respect to the RPE, has been shown to be a fair indicator of the final orientation of the cleavage plane (Chenn et al., 1998; Cayouette et al., 2001).
had no significant effect on the number of vertical divisions, compared with controls (86±2%, N=7). 88±2% of divisions were vertical in controls (N=7) compared with 86±2% following the application of either AMPA (N=6; U=7, P=0.31) or NBQX (N=6; U=12, P=0.43). Thus, purinergic, muscarinic, GABAergic and glutamatergic agonists and antagonists had no significant effect on the number of cells undergoing 'vertical', as opposed to 'horizontal', divisions following their application in ovo.

**Part III. The RPE may regulate mitosis in the neural retina via ATP release**

Purinergic agonists and antagonists influence the speed of mitosis in cells in the VZ (see section 4.3.3). The RPE has been implicated in regulating proliferation in the neural retina (Raymond and Jackson, 1995; Iliia and Jeffery, 1996, 1999) and recent investigations have shown that ATP is released from the retinal but not the choroidal surface of human RPE cells grown in culture (Mitchell, 2001). It is possible that ATP released from the RPE affects the rate of the cell cycle in the neural retina. Hoechst 33342 labelled E5 retinae were imaged under the following conditions: i) in control solution with RPE intact, ii) in control solution with the RPE removed, iii) in the presence of suramin (25μM) with RPE intact, iv) in the presence of suramin with RPE removed, v) in the presence of the ATPase, apyrase (80U/ml), with RPE intact or vi) in the presence of apyrase with RPE removed.

**4.3.10. Mitosis is faster when the RPE is present**

In control preparations in which the RPE was removed (RPE') the average length of time spent in metaphase was 36±2mins (N=4, n=100). In contrast, imaging of the contralateral eye in the presence of the RPE (RPE⁺) showed this time was significantly reduced, to 14±3mins (N=4, n=100, P<0.01 paired Student’s t-test) (Figure 4.18). When RPE⁺ retinae were exposed to UTP (10μM) the time spent in metaphase was reduced to a period similar to that seen in RPE⁺ retinae (12±2mins; N=6, n=99, P<0.01 unpaired Student’s t-test). The application of UTP to RPE⁺ retinae did not further increase the speed of mitosis (11±3mins; N=3, n=75, P=0.67 unpaired Student’s t-test) (Figure 4.19a).
The length of time VZ cells spend in metaphase in RPE\(^+\) retinae is very similar to that seen following the application of UTP in RPE\(^-\) retinae. To test whether the increase in the rate of mitosis observed in the presence of the RPE involves purinergic stimulation of dividing cells in the VZ, suramin (25\(\mu M\)) was applied to retinae both with and without RPE (Figure 4.19b). In RPE\(^-\) retinae, suramin increased the time spent in metaphase but this difference was not statistically significant (37±2mins versus 31±1mins in controls; N=4, n=63, P=0.56 unpaired Student’s t-test). In contrast, in RPE\(^+\) retinae, VZ cells divided at a much slower rate in the presence of suramin compared to their respective controls. In control RPE\(^+\) preparations metaphase lasted 12±3mins (N=6, n=117) compared to 41±10mins (N=6, n=46, P<0.01 unpaired Student’s t-test) in those RPE\(^+\) retinae exposed to suramin.

4.3.11. Apyrase slows the rate of mitosis when the RPE is present

The actions of suramin in slowing mitosis in the presence of the RPE suggest that endogenous ATP, perhaps released from the RPE, may act to promote retinal cell division. Apyrase is an ATPase that breaks down extracellular ATP. Control retinae, either with RPE or without RPE were incubated for 20mins in normal Krebs’, and perfused with the same solution during the imaging period. The corresponding contralateral eyes (RPE\(^+\) or RPE\(^-\)) were incubated in Krebs’ solution containing apyrase (80U/ml, where 1 unit liberates 1.0\(\mu\)mole of inorganic phosphate from ATP or ADP at pH6.5 at 30°C) for 20mins, and then perfused with apyrase-containing Krebs’ during the imaged period. Application of apyrase caused the mitotic activity of VZ cells to fall markedly, preventing many cells from progressing beyond metaphase (Figure 4.19b). In RPE\(^+\) retinae perfused with control solution, all cells that were in metaphase at the start of the recording progressed into anaphase by the end of the 90min imaging period. The same proportions of cells (although not the same cells) were in prophase or metaphase at the end of the experiment as at the outset (4±1 cells versus 3±1 cells/field of view [18x10\(^3\)\(\mu m^2\); N=3, n=1507]. During the 90mins period of the experiments, 99±10 cells/18x10\(^3\)\(\mu m^2\) progressed through prophase to metaphase and finally divided. However, in RPE\(^+\) retinae treated with apyrase, the proportion of cells in prophase or metaphase at the end of recording was raised to 40±9cells/18x10\(^3\)\(\mu m^2\) (N=3, n=1316, P<0.01 paired Student’s t-test). These cells underwent chromosome condensation but
failed to progress beyond either prophase or metaphase. In 3 preparations treated with apyrase only 4 cells (<2 cells/ 18x10^3 μm^2) passed through all stages of mitosis during the imaging period and the time spent in metaphase ranged from 27 to 70 minutes.

Similar effects were seen following the application of apyrase in RPE^- retinal. The proportion of cells that failed to progress beyond metaphase by the end of the recording period increased to 30±1 cells/18x10^3 μm^2 (N=3, n=1504, P<0.01 paired Student’s t-test compared with RPE^- controls). In two of the RPE^- preparations treated with apyrase only 4 and 7 cells, respectively, passed through all stages of mitosis during the recording period and the time spent in metaphase ranged from 18 to 78 mins. In the third preparation examined 30 cells did progress from prophase through to anaphase and took an average time of 29±1 mins.
4.4 Discussion

Cells of the developing neural retina divide at the ventricular surface of the tissue, a feature that allows the use of time-lapse confocal microscopy to follow the mitotic process in situ and in real time. This has been used to demonstrate two findings. Firstly, metaphase rods are in constant rotation within the plane parallel with the VZ. The total time spent in rotation is dependent on the length of time in metaphase and is affected by muscarinic and purinergic stimulation. The onset of anaphase brings about an arrest of this rotational movement. Secondly, purinergic and muscarinic stimulation have opposing effects on the speed at which cells progress through mitosis, whilst GABA and glutamate are without effect. Muscarinic stimulation acts as a brake on mitosis at metaphase, almost doubling the time it takes for chromosomes to separate, while purinergic stimulation acts as an accelerator, reducing the time taken for this process to a third of that in controls. These effects of muscarinic and purinergic stimulation do not appear to be compensated for elsewhere in the cell cycle since the presence of UTP, PPADS, CCh and pirenzipine, in ovo, for a longer period have marked effects on both eye size and the number of dividing cells in the VZ, consistent with the effects of these agents on mitosis.

4.4.1 Rotations of the metaphase plate

Metaphase rods rotate within the plane of the VZ throughout metaphase, making repeated changes in the direction of their rotation. Rotation ceases upon entering into anaphase and the chromosomes pull apart and the cell divides at the angle set at the onset of anaphase. Metaphase rods make large rotations, sometimes in excess of 360°, before changing direction. However, for the majority of the time the rods rotate within the range of 0±45°, where 0° is the angle at which the daughter cells will separate in anaphase. This implies that there is some specification for the orientation of the mitotic apparatus within the plane of division, once the decision to divide vertically or horizontally has been made. During the first stages of development, the majority of PC divisions are vertical (Hinds and Ruffet, 1971; Chenn and McConnell, 1995), but as development progresses, there is an increase in the number of horizontal divisions (Chenn and McConnell, 1995). It follows that there exists some mechanism for
orientating the mitotic spindle with respect to the apical/basal polarity of the PC in order to mediate either a vertical or a horizontal division. Cells were rarely observed 'flipping' between the very distinctive rod- and disk-like appearances of the mitotic apparatus (see Figure 4.1) following their entry into metaphase. This may mean that the decision to divide vertically or horizontally is made before the onset of metaphase, although it could also reflect the fact that the majority of cells are undergoing vertical divisions at this time. Indeed, the number of cells in which the metaphase apparatus displayed a disk-like, rather than a rod-like, appearance was low at the time investigated, consistent with the notion that vertical divisions predominate during the early stages of development (Chenn and McConnell, 1995). However, having started a vertical division, cells spend considerable time apparently “searching” for the correct location within the plane, and this movement stops immediately upon entering anaphase. Similar rotational movements have been observed in dividing PCs in the rat neocortex (Adams, 1996; Haydar et al., 2001) where the metaphase apparatus also undergoes large excursions around a central axis, within the plane of the cortical VZ, with the movement ceasing upon entering into anaphase. In budding yeast, the mitotic apparatus undergoes similar see-saw like movements in a ‘search and capture’ process, until apparently finding the appropriate binding sites for division (for review, see Jan and Jan, 2001). The correct alignment of the mitotic spindle, with respect to either a vertical or a horizontal division, has been associated with the subsequent determination of fate as a result of the segregation of cytoplasmic factors between the resulting daughter cells (Chenn and McConnell, 1995; Chenn et al., 1998, but see below). However, the functions of rotations within the plane of a division (e.g. within a vertical division) are unclear. It is possible that cells dividing vertically inherit as yet unknown factors asymmetrically, perhaps by virtue of interactions with their neighbours, that could predispose one daughter cell to a particular outcome or fate. Cells within a dividing epithelium may also need to co-ordinate division with adjacent cells in order to maintain the integrity of cell-cell contacts during cytokinesis (Hinds and Ruffet, 1971; Reinsch and Karsenti, 1994). Such processes would only be observed in intact preparations such as the retinal whole-mount used here. There were no obvious patterns of division and metaphase cells divided apparently randomly with respect to their neighbours and to other mitotic cells. However, more complex anatomical factors and cell-cell interactions, such as gap junctional coupling (see Chapter 6) that were not
visualised in this imaging process could direct the rotations of the metaphase apparatus and the choice of the angle at which to divide.

Muscarinic and purinergic agonists have profound effects on the time dividing cells spend in metaphase (see below). Purinergic stimulation causes a marked decrease in the length of time spent in metaphase while muscarinic stimulation significantly extends this period. Given that the mitotic apparatus of dividing cells is in constant rotation, the length of time a cell remains in metaphase affects the total extent of rotational movement. Consequently, purinergic stimulation reduces the extent of the rotational movements of the mitotic apparatus while muscarinic stimulation increases it. The functional significance of such rotational movements is not understood, and hence the physiological consequences of shortening or prolonging their duration by purinergic or muscarinic stimulation, respectively, are unknown.

4.4.2 Muscarinic and purinergic, but not ionotropic glutamatergic or GABAergic, receptors regulate the cell cycle

The results presented here provide evidence that P2 receptors and mAChRs are involved in the control of retinal cell proliferation, albeit that some of this evidence is from crude experiments that expose the entire embryo to drugs. Short-term application of UTP reduced the length of time spent in mitosis by cells in retinal whole-mounts. Provided these changes are not compensated elsewhere in the cycle, a concomitant increase in the number of mitotic figures would also be predicted (Rakic, 2002); prolonged exposure in ovo increased eye size and the overall number of dividing cells present in the VZ. Purinergic antagonists extended the time taken to complete mitosis while prolonged exposure in ovo led to a reduction in eye size and the number of dividing cells in the VZ. These results are in good agreement with those of Sugioka et al. (1999b) who showed that ATP and PPADS caused an increase and a decrease, respectively, in the level of $^{3}$H thymidine incorporation in E3 chick retinal organ cultures. Suramin had strong effects, completely inhibiting $^{3}$H thymidine incorporation following exposure for 24h. A possible reason for the very pronounced effects seen by Sugioka et al. and the high mortality rates seen in the in ovo experiments in this chapter, is its potential interference with growth factors. Suramin has been reported to inhibit growth factors
from binding to appropriate receptors (Cirrillo et al., 2001). However, the effects of Reactive Blue, PPADS and UTP all point to the involvement of P2 receptors in the promotion of mitosis and PC proliferation. ATP applied to retinal cell cultures increases proliferation via an extracellular signal-regulated kinase cascade: Sanches et al. (2002) observed an age-dependent ATP-induced increase in both phosphoinositide turnover and incorporation of $^3$H thymidine, effects that were blocked by PPADS (the effects of ATP were less at later times). In agreement with these observations, the effects of UTP on the speed of mitosis were absent when applied to E7-8 retinae. The absence of purinergic receptors linked to the production of $[Ca^{2+}]$, transients at these later times (see Chapter 3) may explain this result and that of Sanches et al. (2002).

Muscarinic agonists have been reported to be able to both stimulate and inhibit proliferation (e.g. Nicke et al., 1999). Muscarinic agonists extended the time retinal PCs spent in metaphase during mitosis, compared with controls. Prolonged exposure in ovo led to a decrease in the number of mitotic figures and an overall reduction in eye size. Conversely, embryos treated with pirenzipine in ovo presented large eyes and an increased number of mitotic figures. Mitotic cells in retinal whole-mounts progressed through metaphase faster in the presence of pirenzipine than in controls. Muscarinic stimulation has been shown to reduce proliferation in a number of cell types (Conklin et al., 1988; Williams and Lennon, 1991; Nicke et al., 1999; Baumgold and Dyer, 1994) and CCh reduced $^3$H thymidine incorporation in E8 chick retinal cultures (Sanches et al., 2002). In contrast, mAChRs have been reported to stimulate the proliferation of cultured cortical astrocytes (Ashkenazi et al., 1989) and PCs (Li et al., 2001; Ma et al., 2000). Whether mAChRs exhibit a stimulatory or inhibitory effect on cell proliferation is likely to depend on their subunit composition and the second messenger and intracellular signalling mechanisms to which they are coupled.

An increase in cell death could account for the observed decrease in the number of mitotic cells and reduced eye size. However, examination of Hoechst-stained retinal sections showed no change in the number of pyknotic nuclei in the retina. Furthermore, no changes were observed in the density of cells within retinae treated with drugs. It is very unlikely that changes in eye diameter or the number of mitotic cells resulted from a difference in the PC population caused by increased cell death or by changes in cell volume. Exposing embryos to neurotransmitters and their antagonists early in
development will undoubtedly affect many developing organs as well as proliferation and differentiation in parts of the nervous system other than the eye. It is difficult to restrict drugs applied to the eye in ovo at E4-6 since the BRB is not tight until after E15 (for review, see Kneisel and Wolburg, 1993) and thus, prior to this time, ocularly applied drugs are able to pass into the bloodstream. Furthermore, mechanical disruption of the eye (e.g. by injection) at these early stages causes severe impairment in eye growth (M. Catsicas, personal communication). Despite the limitations of the technique used here, the congruence of the changes in eye size and level of mitosis with those of the speed of mitosis make them worthy of report.

P2 receptor antagonists slowed mitosis beyond that seen in controls, whilst mAChR antagonists increased the speed of progression through metaphase, results that suggest that endogenous ATP and ACh activate P2 and mACh receptors. That this is so is supported by experiments in Chapter 3, which demonstrated that spontaneous [Ca^{2+}], activity is increased by the inhibition of the breakdown of ACh by iso-OMPA and eserine, and reduced by increased degradation of ATP by apyrase. Further, ChAT immunoreactivity is present at E5 (see Chapter 3) and AChE is present in the chick retina as early as E3 (Layer et al., 1989). Amacrine cells may be a source of ACh at these early times since they are born from E4 onwards (Prada et al., 1991) and a subset, the starburst amacrine, is known to be cholinergic in the adult retina (Famigliette, 1983 review; Hayes, 1984; Voigt, 1986). ATP is co-released with several neurotransmitters including ACh (see Burnstock, 1999). Stimulus-evoked ATP release has been demonstrated in several non-neural cell types including Müller cells (Newman, 2001) and the RPE (Mitchell, 2001). Sugioaka et al. (1996) have shown that ATP is released from retinal cultures and whole embryos; increased [ATP]o was detectable in both culture media and amniotic fluid during early development (E3-5). The maximum concentration of ATP they detected in retinal cultures by luciferin-luciferase assays was 50nM, which is low for receptor activation (although VZ cells showed changes in [Ca^{2+}], in response to 100nM UTP, see Chapter 3) but they estimated a maximum of 3μM in the amniotic fluid of E3 embryos. If the embryonic chick RPE releases ATP into the subretinal space adjacent to the VZ, as suggested by the experiments of Mitchell (2001) on cultured human RPE cells, then the concentration of ATP within the
VZ could be quite high. Alternatively, since the BRB develops after E15 (Kneisel and Wolburg, 1993), both ACh and ATP could also originate from sources outside the eye.

Lo Turco et al. (1995) show that, in slices of embryonic rat neocortex, GABA and glutamate decrease the number of embryonic cortical cells synthesizing DNA and that GABA and glutamate receptor antagonists increase DNA synthesis, indicating that endogenously released amino acids influence neocortical PCs in the cell cycle. They suggest that GABA and glutamate bring about their actions through mechanisms involving depolarisation-evoked $[\text{Ca}^{2+}]_i$ increases, although the identity of the cells responding to these agonists was not established. The findings reported here show that, in the chick retina, the actions of GABA and glutamate (or AMPA) are also to produce increases in $[\text{Ca}^{2+}]_i$ but that these responses are largely restricted to the interphase, and at least for GABA the differentiated, cell population (see Chapter 3 and Pearson et al., 2002), and in contrast to the findings of LoTurco et al. (1995), the $\text{Ca}^{2+}$ influx through VGCCs that they evoke does not appear to regulate mitosis at least during the first week of development. An interesting possibility is that in the neocortex, the actions of GABA and glutamate may be to depolarise differentiated neurons and bring about the release of some factor, such as ACh, which then acts as a brake on the cell cycle. However, this does not appear to be the case in the retina for both glutamatergic, and GABAergic, stimulation and blockade, were without effect on mitosis or eye development at the age examined. Nevertheless, since transmitter systems interact to regulate early electrical activity (Wong et al., 2000), at times both before neurogenesis is complete and synapse formation has occurred (Catsicas et al., 1998), and the activation of GABAergic and glutamatergic receptors becomes more prevalent later on, this possibility is worthy of further investigation. The imaging experiments reported in Chapter 3, and those by Sakaki et al. (1996), show that the purinergic and muscarinic-induced $[\text{Ca}^{2+}]_i$ changes are maximal during the first week of development in the chick, whilst those induced by GABA and glutamate increase rapidly during the second week (Allcorn et al., 1996, Allcorn, 1996). In order to eliminate the possibility that neither GABA nor glutamate regulate proliferation in the chick retina, it will be necessary to look at later times as well as those examined in this thesis. The absence of an effect caused by agonists could also be explained by receptor desensitisation. Whilst embryos were exposed to the agonists for 8h it cannot be assumed that the receptors were able to respond to the agonists throughout this period and it is likely that desensitisation occurs. However,
since GABA and glutamate antagonists were also without effect, the results suggest that
GABA_A and AMPA/kainate receptors have little effect on retinal cell proliferation.

4.4.3 \([Ca^{2+}]_i\) and the cell cycle

How do neurotransmitters exert their effects upon the cell cycle? Owens and Kriegstein
(1998) put forward the hypothesis that \([Ca^{2+}]_i\) activity can influence the cell cycle in the
embryonic CNS. Data presented in this chapter shows that progression from prophase
through metaphase to anaphase is strongly affected by purinergic and muscarinic
receptors, which generate conspicuous \([Ca^{2+}]_i\) activity in VZ cells. Indeed, there is a
striking temporal correlation between the purinergic enhancement of proliferation
shown in these experiments and the mobilization of \(Ca^{2+}\) by ATP and UTP observed in
retinal PCs (Chapter 3; Yamashita and Fukuda, 1993). Furthermore, depletion of
intracellular \(Ca^{2+}\) stores by prior incubation with caffeine, or chelation of cytoplasmic
\(Ca^{2+}\) with BAPTA, both strongly inhibit the progression through mitosis. It is tempting
to speculate that the transmitter-evoked release of \(Ca^{2+}\) from stores within PCs exerts a
direct effect on the rate of mitosis since \([Ca^{2+}]_i\) signals have been shown previously to
be important in controlling the cell cycle (for review, see Whitaker and Larman, 2001).
If this were so, it would provide a mechanism by which molecules such as
neurotransmitters could regulate cell division and exert control over both cell number
and the timing of the production of the different types of CNS cells. \(Ca^{2+}\) has been
shown to be essential in the progression through several steps in the proliferative cell
cycle including the \(G_1/S\) phase transition, progression through several stages within \(S\)
phase itself, entry into mitosis and key points within mitosis such as the metaphase-
anaphase transition and induction of cytokinesis (see Whitaker and Larman, 2001).
Injection of micromolar \(Ca^{2+}\) promotes mitosis, whilst chelation of \([Ca^{2+}]_i\) decreases or
halts its progression. Similarly, changes in the level of \(IP_3\), which releases \(Ca^{2+}\) from
intracellular stores, has been shown to correlate with the resumption of the cell cycle in
fertilized sea urchin eggs (for comprehensive reviews, see Santella, 1998 and Santella et
al., 1998). \(Ca^{2+}\) activates \(Ca^{2+}\)-binding proteins such as the key \(Ca^{2+}\) signal decoder
calmodulin, which stimulates the kinase CaM kinase II. CaM kinase II is essential in the
transitions from \(G_2\) to mitosis, metaphase to anaphase and \(G_1\) to \(S\) phase (see Santella,
1998). Stimulation of a variety of G protein coupled receptors causes stimulation of
MAPKs and PI-3 kinases. These have widespread effects and are involved in relaying
signals to the nucleus, the activation of transcription factors such as c-jun and CREB, the activation of cytoskeletal proteins and the progression from G1 to S phase.

The pathway by which muscarinic agonists cause a decrease in mitosis is unknown but it may also involve the release of Ca\(^{2+}\) from intracellular stores. Calcium ions can have both positive and negative effects on proliferation depending on the cell type and/or the stage of cell cycle. For example, depolarisation and concomitant Ca\(^{2+}\) entry can cause a decrease in DNA synthesis in cortical PCs (for review, see Barker et al., 1998) and depolarisation-induced Ca\(^{2+}\) influx is correlated with both the promotion of progression from G2 to mitosis, and the inhibition of the transition between G1 and S phase in pituitary cells (Ramsdell, 1991). However, Ca\(^{2+}\) may not be the primary effector of the muscarinic response. Sanches et al. (2002) observed similar increases in IP\(_3\) turnover following both purinergic and muscarinic stimulation (although they did not measure any concomitant rise in Ca\(^{2+}\)), but only purinergic agonists caused an increase in proliferation. Muscarinic receptors can link to downstream effectors other than Ca\(^{2+}\) including cAMP and DAG. The muscarinic response could be mediated by various kinases and phosphatases, activated via some combination of these 2\(^{nd}\) messengers.

The inhibitory effects of chelating cytoplasmic Ca\(^{2+}\) and of depleting intracellular Ca\(^{2+}\) stores upon progression through metaphase shown in this chapter support the notion that the appropriate [Ca\(^{2+}\)]\(_i\) signals are necessary for the mitotic process to progress. The existence of Ca\(^{2+}\)-dependent processes in mitosis has only recently gained acceptance. If [Ca\(^{2+}\)]\(_i\) signals are pivotal to the regulation of cell proliferation in the retina then the presence of several neurotransmitter receptor systems, the prevalence of which changes with time, linked to [Ca\(^{2+}\)]\(_i\), changes suggests that neurotransmitter receptors could play a more complex role than simply controlling the rate of PC division.

**4.4.4 Receptors can use the same downstream effectors to cause different effects**

Both muscarinic and purinergic agonists induce increases in [Ca\(^{2+}\)]\(_i\) via release from intracellular stores. It is difficult to understand how two similar [Ca\(^{2+}\)]\(_i\) signals could exert opposing effects on mitosis. One explanation may be that Ca\(^{2+}\) is not involved in mediating one, or perhaps either, of the effects of purinergic and muscarinic stimulation on mitosis but rather some other 2\(^{nd}\) messenger is involved (see above). However, Ca\(^{2+}\)
signalling is both spatially and temporally regulated and can thus control multiple processes within individual cells. \([Ca^{2+}]_i\) signals range from very localized, sub-cellular, single transients to large oscillations involving the whole cytoplasm. The pattern of these signals and their cellular extent depends upon the mechanisms of release, amplification and the degree of spatial buffering (for review, see Røttingen and Iversen, 2000). The amplitude, duration and kinetics of \([Ca^{2+}]_i\) signals have all been shown to cause the differential activation of transcription factors (Dolmetsch et al., 1998; West et al., 2001). In Chapter 3, \(Ca^{2+}\) imaging experiments indicated that doses of UTP and CCh, sufficient to evoke a response in 50% of the VZ population, result in changes in \([Ca^{2+}]_i\) that had different magnitudes and produced oscillations in \([Ca^{2+}]_i\) of different frequency.

The opposing effects of mAChRs and P2 receptors on mitosis could also arise from the existence of two or more populations of PCs. Recent studies in the cortex have indicated that significant differences exist between neural PCs during the early development of the forebrain. Lineage-dependent differences between cells allow subpopulations of cells to selectively respond to environmental cues (Eagelson et al., 1998). However, Sakaki et al. (1996) and Sanches et al. (2002) both observed that successive applications of CCh attenuates the retinal \([Ca^{2+}]_i\) response to a subsequent application of ATP, and propose that this results from a CCh-induced depletion of intracellular \(Ca^{2+}\) stores that prevents a maximal response to ATP, a result that suggests that the two receptors are expressed in the same cell. The imaging experiments in Chapter 3 demonstrated that individual cells can respond to both muscarinic and purinergic stimulation; \(>90\%\) of PCs in the VZ responded to both CCh and UTP at E4. Thus, PCs are likely to express both receptors (although the possibility remains that stimulation with either agonist leads to release of a \(2^{nd}\) signal that causes \([Ca^{2+}]_i\) to rise in neighbouring PCs). The down stream effects of a G-protein coupled receptor depend on which G-protein it interacts with and where in the cell the receptor is localized. The Xenopus oocyte presents an asymmetric distribution of both the numbers of mAChRs and the subsequent functional response (Matus-Leibovitch et al., 1990; Davidson et al., 1991). Nadler et al. (1999) used a MDCK (Madin-Darby Canine Kidney) cell system to show that different subtypes of mAChR may be targeted to different regions of polarized cells. A similar situation could exist in retinal PCs and could be investigated by local application of drugs onto isolated cells. Further diversity occurs within the intracellular
Ca\textsuperscript{2+} stores themselves. The sensitivity of IP\textsubscript{3} receptors is dependent upon the isoform and splice variant expressed, ambient Ca\textsuperscript{2+} and ATP levels, and whether or not the receptor is phosphorylated. Thus, two signals can mediate IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release from different intracellular stores. This has been demonstrated for HEK (Human embryonic kidney) 293 cells where CCh and ATP mobilize Ca\textsuperscript{2+} from independent IP\textsubscript{3}-sensitive stores (Short et al. 2000). Thus, there exists numerous ways in which extracellular signals can utilise [Ca\textsuperscript{2+}]\textsubscript{i} transients to achieve different down stream events.

4.4.5 Symmetric and asymmetric division

The orientation of the mitotic spindle at division has been associated with the subsequent determination of fate of the two daughter cells in vertebrates and invertebrates (Chenn and McConnell, 1995; for reviews see Rhyu and Knoblich, 1995, Jan and Jan, 2001). When there is a requirement for expansion of the PC pool, as at the start of development, the proportion of proliferative, symmetrical divisions is high. As neurogenesis progresses, there is an increase in the number of asymmetric neurogenic divisions and a concomitant decrease in the number of proliferative divisions (Caviness et al., 1995; Takahashi et al., 1994, 1995). Late in development, the number of proliferative divisions reduces as PCs switch to symmetric terminating divisions, which each produce two NDCs. Asymmetric inheritance of several potential cell-fate determinants, such as Notch and Numb, has been demonstrated for mammals and chicks (e.g. Chenn et al., 1998; Cayouette et al., 2001). On the basis of correlative anatomical studies, Chenn and McConnell (1995) proposed that the orientation of the cleavage plane, with respect to the cortical plane, determines whether a division is symmetric or asymmetric. In this chapter, the orientations of dividing cells were measured in order to examine firstly, the distribution of cleavage planes in the early chick retina and secondly, whether neurotransmitters applied \textit{in ovo} affected the orientation of division. In control preparations cells divided with their mitotic apparatus orientated at all angles between the vertical and the horizontal but the majority occurred at or very near to the vertical position. That a continuum, rather than a bimodal distribution, of angles between the vertical and horizontal orientation was found, is perhaps concerning for a hypothesis that proposes the plane of cleavage is fundamentally involved in determining asymmetric inheritance of fate determinants. Indeed, the angle that determines the cut-
off between symmetric and asymmetric divisions has been variously taken as 10° to 45° away from the vertical by different authors (Chenn and McConnell, 1995; Chenn et al., 1998, Cayouette et al., 2001). In the experiments presented here an angle of 45° was used as the arbitrary cut-off between ‘vertical’ and ‘horizontal’ divisions. Under the hypothesis put forward by Chenn and McConnell (1995), manipulations that actively promote proliferation might be expected to correlate with a proportion of symmetrical and, hence vertical, divisions. Manipulations of purinergic, muscarinic, glutamate and GABAergic receptors had no obvious effect on the distribution of divisions. As in controls, cells were found dividing at all angles, with no change in either the distribution of angles or the proportion of ‘vertical’ versus ‘horizontal’ orientations. Experiments described above show that purinergic stimulation increases both mitosis and the number of dividing cells in the VZ, and consequently produces bigger eyes. These changes indicate that proliferative divisions are enhanced by purinergic stimulation. However, no corresponding increase in vertically orientated divisions was found. It is possible that purinergic stimulation of proliferation induces cells to exit the proliferative cycle prematurely. A DNA-binding motif protein called Pax6 is involved in the regulation of the cell cycle. Estivill-Torrus et al. (2002) demonstrated that cortical PCs in mice lacking functional Pax6 presented an initial increase in the level of proliferation and an increase in the proportion of horizontal to vertical divisions, and proposed a premature enhancement of asymmetric divisions. However, this does not seem to be the case in the retina, as horizontal divisions occurred at the same frequency in all experimental conditions and in controls. Similarly, slowing of the cell cycle via muscarinic stimulation had no discernible effects upon the orientation of the cleavage plane over the 8h period investigated here. Experiments in the cortex indicate that GABAergic and glutamatergic receptors are expressed during the final cell cycle (Maric et al., 2000; Maric et al., 2001) and thus, in theory, have the potential to influence the orientation of division at this time. However, agonists and antagonists of both receptors had no effect upon the orientation of the cleavage plane. Again, it is likely that prolonged exposure to an agonist will cause receptor desensitisation and hence the absence of effect on the angle of division caused by agonists does not necessarily mean that these receptors have no influence on the cleavage plane. However, the absence of an effect of any of the antagonists on the orientation of division (whilst affecting eye
size and mitosis), suggests that the orientation of division is not influenced by mAChR, P2, GABA_A or AMPA/kainate receptors.

Very recent evidence has called into question the functional significance of cleavage plane orientation. Silva et al. (2002) demonstrated that the frequency of vertical and horizontal divisions was the same in regions of the chick retina containing differentiating cells as in regions in which only proliferative divisions were occurring. Horizontal cleavages are apparently capable of producing two PCs. Similarly, these authors found no distinction between vertical and horizontal divisions, observing a continuum of orientation biased towards a vertical division. Furthermore, the antigen RA4, an early marker of differentiating RGCs (Waid and McLoon, 1995), was present in cells demonstrating mitotic figures at every possible orientation. In contrast, Chenn and McConnell’s hypothesis would predict that RA4 would only be seen in the basal pole of cells dividing horizontally. If the orientation of cleavage plane does not determine the decision to exit the cell cycle, it raises the question of what function, if any, the different angles of divisions serve.

4.4.6 Developmental consequences of purinergic and muscarinic regulation of proliferation

The regulation of cell cycle dynamics is considered to be a contributing mechanism in the generation of cell diversity. PCs initially cycle rapidly, dividing to increase their number. The cell cycle then progressively slows down as neurogenesis proceeds (Caviness et al., 1995). The expression of mAChR and P2 receptors is temporally correlated with a period of intense proliferation and differentiation in the retina. Several investigations have shown that the purinergic response is maximal during early development (see Chapter 3; Sakaki et al., 1996; Sugioka et al., 1996), while the mAChR responses, while prevalent at early times, are sustained until at least E6-7 (see Chapter 3; Yamashita et al. 1994; Sakaki et al. 1996). During early development, purinergic stimulation could act to promote rapid cycling, thereby increasing the number of PCs in the progenitor pool. This hypothesis is supported by the observation that purinergic stimulation causes an increase in eye size. However, it is not known whether there is any compensation for this effect at later times (see below). Later in eye development, a decrease or down-regulation of the purinergic signal could allow the
muscarinic signal to exert a stronger effect, producing a slowing of the PC cycle. In the chick, the highest period of retinal neurogenesis occurs circa E6 (Prada et al., 1991). The experiments described in this chapter do not address the long-term consequences of mAChR or P2 receptor stimulation. It is possible that inducing increased proliferation early in development has the effect of reducing it later on. Although Sugioka et al. (1999b) found increased levels of $^3$H thymidine incorporation in E3 retinal organ cultures after 24h exposure to ATP, Ciccarelli et al. (1994) describe time-dependent effects of ATP on rat astrocyte proliferation. 8h application of ATP promoted proliferation, while a 16h exposure resulted in decreased proliferation.

The competence model is one of the presiding theories of retinal development. It proposes that PCs pass through a series of competence states, during each of which PCs are competent to produce a subset of retinal cell types (Cepko et al., 1996). The different competence states appear to be determined by intrinsic mechanisms. Within a given competence state, the generation of a particular cell type is modulated by positive and negative extrinsic signals (Cepko et al., 1996, for review, see Livesy and Cepko, 2001). Little is known about the intrinsic changes and a key question is how a PC moves between competence states. One possibility is that environmental signals might drive cells through competence states (Cepko et al., 1996). Alternatively, some form of intrinsic motor or clock (Temple and Raff, 1986) may predetermine at what point cells move between them. If the decision to move through a competence state is determined by the number of cycles of division a PC has passed through, then changing the cell cycle time of a group or population of PCs will affect the time at which post mitotic cells are produced. Since neuronal identity is dependent, at least partly, upon the time of cell birth, a premature exit from the cell cycle could lead to enhanced number of early born neurons. Conversely, delayed exit may lead either to the delayed production of early cell types or, possibly, an increase in later born types of neurons at the expense of early born ones. However, it is possible that other mechanisms would act to prevent these outcomes. RGCs have been shown to self regulate their production by inhibiting the production of new RGCs and by killing incoming RGCs (Waid and McLoon, 1998). Alternatively, rapid cycling of PCs in the early stages of development could enhance the number of PCs in a similar competence state. These may then move in synchrony through the different states as a result of developmentally changing extracellular cues. It
will be of particular interest to determine whether early enhancement of proliferation promotes a bias towards a given fate.

Since the VZ is the only place within the developing retina that PCs divide, it is possible that muscarinic and purinergic receptors may play a further role in the control of the cell cycle through regulating the rate at which cells undergo INM and reach the VZ. Alternatively, it is possible, since \([\text{Ca}^{2+}]_i\) transients have been shown to occur during other phases of the cell cycle (Santella, 1998), that muscarinic and purinergic stimulation of these during the G/S transition and during S-phase which occur outside the VZ, regulate the rate of proliferation. In order to determine whether or not neurotransmitter-evoked \([\text{Ca}^{2+}]_i\) signals affect INM, it is necessary to image living cells as their nuclei move through the retina. The nature and regulation of these movements are investigated in Chapter 5.

4.4.7 Potential RPE-mediated purinergic regulation of mitosis

The final part of the work reported above investigated the influence of the RPE on the speed of mitosis in dividing retinal PCs. Recent experiments have shown that factors secreted by the RPE can influence the normal organogenesis of the neural retina (Sheedlo and Turner, 1996; Jablonski et al., 2000; Aymerich et al., 2001). The results presented here suggest that neurotransmitters may be included amongst these regulatory factors. The presence of the RPE reduces the time dividing retinal PCs spend in metaphase as compared to neural retinal preparations from which the RPE had been removed. It is possible that the slower speeds seen in the isolated neural retinal preparations were due to damage caused by the removal of the RPE. However, this seems unlikely given that application of the purinergic agonist UTP increased the speed of division in these isolated preparations to levels close to that seen in retinae with intact RPE, indicating that the mitotic machinery is at least capable of functioning at its normal rate. An alternative explanation is that the slowing of mitosis results from the absence of some factor(s) or signal(s) following the removal of the RPE and that these can be mimicked, or compensated for, by application of UTP. Additional stimulation of purinergic receptors in the presence of the RPE had little effect on the speed of division. This result could be explained by the release of ATP from the RPE at levels near optimal for the enhancement of the rate of mitosis. The mitogenic effects of the RPE
were antagonised by the inhibition of purinergic receptors with suramin; the time spent in metaphase was extended to levels in the range of those seen following application of suramin to isolated neural retinae. Reducing endogenous levels of extracellular purinergic agonists by the non-specific ectonucleotidase apyrase either massively slowed or prevented progression through the cell cycle. Taken together, the results presented here suggest that the RPE stimulates cell division at least in part through the release of ATP. Mitchell (2001) has shown that ATP release from cultured human RPE cells is polarised and occurs only at the surface adjacent to the neural retina. If a similar release of ATP, or other purine nucleotides, occurs into the subretinal space in the chick retina it could act to drive cell proliferation in the neural retina. However, the neural retina may also be a source of purines; in the adult retina, ATP has been shown to be released from retinal astrocytes (Newman, 2001) and inner retinal neurons (Neal and Cunningham, 1994; Santos et al., 1999).

4.6 Summary

Direct imaging of the mitotic process and examination of the effects of chronic exposure to agonists and antagonists shows that both muscarinic and purinergic stimulation significantly affect the proliferative cell cycle in the retina. Muscarinic stimulation acts as a brake, while purinergic stimulation acts as an accelerator. The effects of these transmitter systems on the cell cycle are dependent upon \([Ca^{2+}]\), and are consistent with the temporal pattern of their actions on \([Ca^{2+}]\), described in chapter 3. It is conceivable that the transmitter-evoked release of \(Ca^{2+}\) from stores within PCs exerts a direct effect on the rate of mitosis. If this is so, it would provide a mechanism by which molecules such as neurotransmitters could regulate cell division and exert control over both cell number and the timing of the progression through the different types of CNS cells. \([Ca^{2+}]\) transients are associated with progression through check points in the cell cycle (see Santella 1998a; Santella et al., 1998; Whitaker and Larman, 2001 for reviews) and are correlated with events such as pronuclear migration, nuclear envelope breakdown, the metaphase-anaphase transition of mitosis, and cytokinesis. calcium Whilst GABA and glutamate are also able to produce increases in \([Ca^{2+}]\), the \(Ca^{2+}\) influx through VGCCs that they evoke does not appear to regulate mitosis during the first developmental week.

Since the VZ is the only place within the developing retina that PCs divide, it is possible that muscarinic and purinergic receptors may play a further role in the control of the cell cycle through regulating the rate at which cells undergo INM and reach the VZ. In order to determine whether or not this is so, it will be necessary to image cells as their nuclei move through the retina during INM. These movements are investigated in chapter 5.
4.5 Further studies

The experiments reported in this thesis investigate the effects of neurotransmitters at early times in development. Further experiments are required to determine their roles at later times.

It would be of great interest to determine the effects of neurotransmitter systems on cell fate. As noted above, muscarinic and purinergic systems could change cell fate via a number of mechanisms. Labelling cohorts of cells dividing in the presence of purinergic or muscarinic agonists with a marker such as BrdU would allow them to be located later in development and assessed for any differences in the proportions of adult cell types produced. Marking individual PCs with GFP would permit clones of cells to be traced and would indicate whether purinergic or muscarinic stimulation affects the number of proliferative divisions an individual PC goes through prior to its terminal division.

The effects of the RPE and purinergic stimulation on mitosis present a potentially novel way for the RPE to promote proliferation in the developing retina. Evidence of apical release of ATP from the embryonic chick RPE would provide strong supporting evidence for the hypothesis that RPE-mediated purinergic release stimulates PC proliferation. A potentially interesting, but technically challenging experiment would be to measure ATP release from the RPE whilst simultaneously imaging the $[Ca^{2+}]_i$ waves that occur in the tissue (see Chapter 6). This could be done using Fluo-4 AM labelling of the RPE combined either with luciferin/luciferase measurements of ATP (Sorensen and Novak, 2001) or the use of a suitable P2X expressing cell held in whole-cell patch clamp mode to act as a sensor for ATP release.
Figure 4.1. Real-time confocal imaging of mitosis. Confocal images of E5 VZ cells labelled with Hoechst 33342. A, Schematic diagram of a section (xz) through the retina showing “vertical” and “horizontal” divisions. The dashed lines indicate the mitotic equator and cleavage plane. Vertical divisions predominate in the E5 chick retina. B, An example of a region in the xy plane of the VZ, showing cells in all stages of mitosis and interphase. Red arrow head: cell in interphase. Small yellow arrow: a cell in prophase. Yellow arrow head: metaphase. Thick yellow arrow: chromosomes separating at anaphase. Thin red arrows: two daughter cells entering interphase following cytokinesis, and before moving away from the VZ. Scale bar 5μm C, Reconstructions of a mitotic and an interphase cell from serial xy sections taken at 0.5μm intervals. Three views are shown: xy, which views the VZ from above (outlined in blue), and xz (outlined in green) and yz (outlined in red) which are sections through the VZ at 90° to one another, taken along the green and red lines shown in the xy image.
Figure 4.2. Time-lapse series of mitosis. Sample images taken from a 90min movie of mitosis in which an E5 retina was labelled with Hoechst 33342 and imaged every 15s. The images are single xy confocal sections taken at the level of the VZ. The chromosomes of a mitotic cell (arrow) progress from prophase (0mins), through metaphase (5 and 25mins) and into anaphase (28 and 30mins). Scale bar 5μm.
Figure 4.3. Rotations of the mitotic apparatus within the plane of the VZ. A, Schematic diagram showing a vertically dividing cell whose mitotic apparatus rotates around a central axis, and flips briefly between the vertical and horizontal orientation (time point 4). B, Time lapse series of a vertically dividing, mitotic cell in the VZ of an E5 chick retina. Retinae were stained with Hoechst 33342 and imaged at 15s intervals for 90mins using time-lapse confocal microscopy. The cell progresses from prophase to anaphase. The metaphase plate is in constant rotation until just prior to the onset of anaphase. The two sets of daughter chromatids continue to separate with little change in the orientation of the spindle. White arrows indicate the orientation of the rod. Scale bar 5μm.
Figure 4.4. Paths of metaphase rod rotation during mitosis in the E5 retinal VZ. Polar traces show the time course of rod reorientation for 5 representative cells. Measurements were taken at 75s intervals during metaphase (filled circles) and anaphase (open circles). Data are presented as polar plots with increasing radius representing progression through time and rod orientation shown relative to the mean angle during anaphase (0°). Right of each polar plot is a “rose diagram” of rod orientations during metaphase. The area of each 30° section is proportional to the time spent at the angles within the bin. Cells A–C preferentially remained at an orientation near that adopted at anaphase whilst cells D and E demonstrated large excursions of more than 180° and 360° respectively.
Figure 4.5. Rose diagrams of the orientations of chromatin during metaphase and anaphase, relative to the mean angle at anaphase (0°) for a population of 20 dividing cells (N=4, E5 retinae). Measurements were separated into those occurring in metaphase (top) and those after the onset of anaphase (bottom). Each data set was binned into 10° intervals and presented in a rose diagram. The area of each sector is proportional to the frequency of observation occurring at the angles within the bin. The mean angle and SD are shown in red. The absence of much rotational movement during anaphase results in a tight clustering of the angles of the separating rods around 0°. The distribution of rod orientations in metaphase is much broader, reflecting the extensive movement of the mitotic apparatus. However, the distributions show a clear preference towards angles near that at which the cells finally divide.
Figure 4.6. Time spent in metaphase is affected by muscarinic and purinergic, but not by GABAergic or glutamatergic, stimulation in the E5 chick retina. Graph of the time (mins) spent in metaphase in either control solution (dark bars) or in the presence of UTP (10μM, N=3), CCh (10μM, N=4), GABA (20μM, N=5) or glutamate (20μM, N=5) (grey bars). Values are mean ± SEM; **P<0.01.
Figure 4.7. Time spent in metaphase is affected by muscarinic but not purinergic inhibition in the E5 chick retina. Graph of the time (in mins) spent in metaphase in either control solution (dark bars) or in the presence of 25µM pirenzipine (N=3) or 25µM suramin (N=4) (grey bars). Pirenzipine significantly decreased this interval (***P<0.01) but suramin was without effect.
Figure 4.8. Eye size is affected by muscarinic and purinergic, but not GABAergic or glutamatergic, receptor activation and blockade in ovo. The effects of UTP (N=4), PPADS (N=4), CCh (N=10), pirenzepine (N=10), GABA (N=6), bicuculline (N=6), AMPA (N=6) and NBQX (N=6) (agonists, 25μM and antagonists, 50μM), applied in ovo for 8h on eye diameter. All embryos were E5 (see Results). Each drug is shown as a percentage of that in control retinae. (Control grey filled bar; agonists dark grey bars; antagonists grey stripes). Values are mean ± SEM; Results were considered statistically significant on one of two levels, *P<0.05, **P<0.01 determined using ANOVA and Dunnett’s test for multiple comparisons against controls. The ANOVA F statistic was significant at P<0.0001 for purinergic and muscarinic drugs.
Figure 4.9. Changes in eye size following muscarinic stimulation or inhibition. Representative light microscope images of the eyes of E5 embryos following *in ovo* application of CCh (~50μM), PBS control or pirenzipine (~25μM) for 8h. Scale bar=5mm.
Figure 4.10. Cell density and cell death are not affected by muscarinic, purinergic, GABAergic or glutamatergic stimulation in ovo, at E5. A-C, Examples of transverse sections through the retina near the optic nerve labelled with Hoechst 33342 to monitor the change in the number of mitotic figures following application, in ovo, of pirenzipine (A), and CCh (C) when compared to control (B). These sections also demonstrate that cell density is unchanged following prolonged exposure (8h) to the same drugs. D, cell death was not affected by application of UTP (N=4), PPADS (N=4), CCh (N=10), pirenzipine (N=10), GABA (N=6), bicuculline (N=6), AMPA (N=6) and NBQX (N=6) in ovo. Graph shows the number of pyknotic nuclei/100µm which have been normalised with respect to controls. P values are shown on the graph. Pyknotic nuclei were absent from the majority of sections examined.
Figure 4.11. Mitosis is affected by muscarinic and purinergic, but not GABAAergic or glutamatergic, receptor activation and blockade in ovo, at E5. The effects of UTP (N=4), PPADS (N=4), CCh (N=10), pirenzipine (N=10), GABA (N=6), bicuculline (N=6), AMPA (N=6) and NBQX (N=6) (agonists: ~50μM, antagonists: ~25μM), applied in ovo, on the number of mitotic figures in the VZ. The effects of each drug have been normalised with respect to controls. Values are mean ± SEM; *P<0.05, **P<0.01. The ANOVA F statistic was significant at P<0.0001 for purinergic and muscarinic drugs.
Figure 4.12. The angle at which a cell divides with respect to the cortical plane results in either horizontal or vertical divisions. A, schematic diagram showing the proposed correlation between vertical/horizontal and symmetric/asymmetric divisions in the cortex (Chenn and McConnell, 1995): a vertical division (left) is thought to produce two proliferative daughter cells, whilst a horizontal division (right) is thought to produce one proliferative daughter cell and one daughter cell that leaves the proliferative cell cycle and differentiates. Dashed lines indicates the orientation of the cleavage plane B, Measurement of the orientation of division. Confocal image of a 20μm section through an E5 retina stained with Hoechst (2μM). The mitotic spindle is assumed to lie at right angles to the metaphase rod. The angle (α) of division was taken as that between a line drawn along the length of the metaphase rod (dotted) and a line drawn perpendicular to the plane of the RPE (shown in red).
Figure 4.13. Orientations of the metaphase rod in dividing PCs. In the majority of dividing cells the metaphase rod was aligned along, or near to, a line perpendicular to the plane of the RPE (black arrow head) i.e. the mitotic spindle lay parallel to the RPE. Such cells are most likely to undergo ‘vertical’ divisions. For small numbers of mitotic cells the metaphase rod was parallel to, and the mitotic spindle perpendicular to, the RPE (small arrow). These cells are thought to undergo ‘horizontal’ divisions. The angle of the metaphase rod of many cells lay somewhere between these two extremes. One example of a cell dividing at an angle of −70° is highlighted (white arrow).
Figure 4.14. The effect of purinergic drugs on the plane of cleavage. The graphs are frequency histograms of the orientations of divisions (as suggested by the orientation of the metaphase rods—see text) in the retinae of E5 embryos injected with either PBS (N=8, top), UTP (50μM, N=4, middle) or PPADS (25μM, N=4, bottom). The pie charts show the proportions of vertical (dark grey) and horizontal (light grey) divisions in each condition, where ‘vertical’ is defined as an angle ≥45°.
Figure 4.15. The effect of muscarinic drugs on the plane of cleavage. The graphs are frequency histograms of the orientations of divisions (as suggested by the orientation of the metaphase rods—see text) in the retinas of E5 embryos injected with either PBS (N=7, top), CCh (50μM, N=10, middle) or pirenzipine (25μM, N=10, bottom). The pie charts show the proportions of vertical (dark grey) and horizontal (light grey) divisions in each condition, where ‘vertical’ was defined as an angle ≥45°.
Figure 4.16. The effect of GABAergic drugs on the plane of cleavage. The graphs are frequency histograms of the orientations of divisions (as suggested by the orientation of the metaphase rods—see text) in the retinae of E5 embryos injected with either PBS (N=7, top), GABA (50μM, N=6, middle) or bicuculline (25μM, N=6, bottom). The pie charts show the proportions of vertical (dark grey) and horizontal (light grey) divisions in each condition, where 'vertical' is defined as an angle ≥45°.
Figure 4.17. The effect of glutamatergic drugs on the plane of cleavage. The graphs are frequency histograms of the orientations of divisions (as suggested by the orientation of the metaphase rods—see text) in the retinas of E5 embryos injected with either PBS (N=7, top), AMPA (50µM, N=6, middle) or NBQX (25µM, N=6, bottom). The pie charts show the proportions of vertical (dark grey) and horizontal (light grey) divisions in each condition, where ‘vertical’ is defined as an angle ≥45°.
Figure 4.18. Mitosis in the VZ progresses at a faster rate in the presence of the RPE (RPE⁺; chequered fill) than in its absence (RPE⁻; solid fill). Histogram showing the time spent in metaphase by dividing cells in the E5 retina. Preparations were imaged either with the RPE intact, or following its removal. Results are shown as mean ± SEM, N=4. **P<0.01, paired t-test
Figure 4.19. Release of a purinergic agonist by the RPE may regulate mitosis in the VZ. A, histogram showing the time spent in metaphase by dividing cells either in the presence (RPE\textsuperscript{+}, chequered fill) (N=4) or absence (RPE\textsuperscript{-}, solid fill) (N=4) of the RPE. E5 preparations both with and without RPE were perfused either with Krebs’ solution (control) (N=4 for both conditions) or with Krebs’ containing the purinergic agonist UTP (10\,\mu M) (N=3 and N=6 respectively). B, histogram showing the time spent in metaphase by dividing cells either in the presence or absence of the RPE. Preparations both with and without RPE were perfused either with Krebs’ solution (control), Krebs’ containing the purinergic antagonist suramin (25\,\mu M; solid or chequered pale grey bars) or Krebs’ containing the ATPase apyrase (80\,U/ml; dotted bars. Note scale bar extends to >90 mins—see text). The number of retinae examined are shown on the graph. Results are means ± SEM. **P<0.01.
Chapter 5

Confocal imaging of interkinetic nuclear migration using a biolistic labelling technique

5.1 Introduction

Our current understanding of dynamic aspects of the PC cycle arises largely from early EM and \(^3\)H-Thymidine labelling studies (Sauer, 1935; Sidman et al., 1959, 1961; Seymour and Berry, 1975; Nagele and Lee, 1979) and more recent observations of labelled ventricular cells using video microscopy (e.g. Chenn and McConnell, 1995). After mitosis, retinal daughter cells extend a process from the VZ to the GCL prior to translocation of the nucleus to the vitreal surface in G\(_1\) of the cell cycle. Following DNA replication in S-phase the nucleus returns to the VZ in G\(_2\) of the cell cycle and the vitreal process retracts prior to mitosis (Figure 5.1). The process of nuclear translocation along the radially extending vitreal or ventricular process during G\(_1\) and G\(_2\), respectively, is termed INM. After a terminal division, NDCs also migrate from the VZ to take up a location appropriate to their fate (Figure 5.1). Little is known about the factors that may regulate INM in the retina. This is partly because it is difficult to label individual cells so that they can be easily distinguished from others and observed in living tissue over extended periods.

Lipophilic carbocyanine dyes can be used to label cells in vivo for long periods without obvious cytotoxicity (Honig and Hume, 1986). Labelling is usually achieved by sprinkling crystals of dye onto the surface of the tissue (e.g. O’ Rourke et al., 1994; Cline et al., 2000). However, this technique gives very variable results because the size and density of the crystals is hard to control. Gan et al. (2000) have pioneered a novel use of the biolistic transfer technique commonly used for GFP-transfection. A ‘gene gun’ is usually used to propel plasmid-coated beads into tissue. When these beads enter cells, they can cause gene expression (Lo et al., 1994). However, in cell culture and brain slices, GFP expression is not evident until ~6h after transfection. In addition, because only cells that have a gene-coated particle in or near their nucleus can be labelled, it is difficult to get high-density labelling without causing damage to the tissue. Gan et al. coated beads with carbocyanine dyes and used a ‘gene gun’ to fire them into cortical slices. This “DiOlistic” method enables the multicolour labelling of many
spatially dispersed cells in a single preparation. Here, the technique has been adapted for use in the developing chick retina.

The aims of the experiments described in this chapter were i) to develop techniques to enable the imaging of migration of PCs and NDCs and the measurement of any correlated $[\text{Ca}^{2+}]_i$ transients, and ii) to investigate the influence of neurotransmitters on the migration of cells. The experiments in Chapter 4 showed that purinergic and muscarinic stimulation acts to, respectively, speed up and slow down, the process of mitosis. It is possible that the $[\text{Ca}^{2+}]_i$ changes seen in the VZ in response to purinergic and muscarinic stimulation directly affect the rate of mitosis. Such $[\text{Ca}^{2+}]_i$ transients might also either speed up or slow down the rate of INM in PCs. The VZ is the only place retinal PCs can divide, and so speeding up or slowing down the speed at which the nuclei of PCs travel to and from the VZ could influence the rate of cell proliferation.
5.2 Methods

The biolistic dye-labelling technique used here has been adapted from a protocol used by Gan et al. (2000). Details of the methods used in this chapter are described in sections 2.4 and 2.7 of Chapter 2. In experiments to examine PC morphology and migration, E5 retinas were biolistically labelled with Dil and DiO and imaged at 36°C. Labelled cells were imaged in the xy plane of the retina and images were taken at 1μm steps, throughout the depth of the retina, and for ~5μm beyond on either side. This was repeated at 15 or 20min intervals. 3-D projections were built from the image stacks and analysed off-line using Metamorph or Kinetic Imaging software. The position of cell nuclei was recorded for each time point. The average speed of migration was determined from the total distance moved by the nucleus over the imaging period.

\[\text{[Ca}^{2+}\text{]}_i\] transients in migrating PCs were assessed by imaging folded, Fluo-4 AM loaded, retinas in the xy plane to create ‘virtual z sections’ (see Chapter 2 for details). Images were taken at 10 or 40s intervals and imaged off-line using Lucida software. A problem in the recording of \([\text{Ca}^{2+}\text{]}_i\) transients in migrating PCs is that if the cell body shape changes during movement, or migrating cells move out of the plane of focus, changes in Fluo-4 intensity may not directly relate to a change in \([\text{Ca}^{2+}\text{]}_i\). In an attempt to partially circumvent this problem a larger pinhole size was used to increase the thickness of the image plane. Ratiometric measurements confirmed that the changes in fluorescence seen using this technique reflect real changes in \([\text{Ca}^{2+}\text{]}_i\) rather than changes in the image plane or cell thickness (see section 2.4.4 and Figure 2.2, Chapter 2).

The distribution of the speeds of cell movement was skewed. In order to perform parametric analysis, all data was transformed to a Lognormal distribution (GraphPad Prism 3.00). Speeds of movement towards and away from the VZ were compared with an unpaired Student’s t-test. Data from drug-treated eyes were compared with that of the contralateral control eye using a paired Student’s t-test. Differences were considered statistically significant at one of two levels: *P<0.05 and **P<0.01. Results are means ± SEM, where N=number of retinas investigated and n=number of cells recorded.
5.3 Results

5.3.1 Labelling retinal cells using the ‘DiOlistic’ technique

The biolistic technique labelled cells at all stages of the proliferative cell cycle. Firing dye-loaded bullets at the ventricular side of the retina labelled a large number of cells that had a PC-like appearance, with an oval cell body and processes extending to the ventricular and vitreal surfaces (Figure 5.2a). The nuclei of individual cells were seen at all depths throughout the thickness of the retina. Particles fired at the vitreal surface labelled many early RGCs but fewer PCs. PCs and their processes, and RGCs and their dendritic trees, appeared fully labelled <4 min after particle delivery onto living E5 retinae (Figure 5.2a and b). Two minutes after particle delivery, multiple cell bodies were labelled and some labelling of the cell’s processes was apparent. Later, labelled processes could be followed throughout the depth (~80|μm) of the retina, and in the case of RGC dendrites, for much greater distances.

Neighbouring cells could be differently labelled red, green or yellow by preparing particles that were coated with a mixture of DiI and DiO (Figure 5.3). Using two excitation wavelengths (488 nm and 568 nm krypton-argon laser lines) and the appropriate filters (522 nm and 590 nm, respectively), cells labelled with DiO only-, DiI only- or DiI and DiO-coated beads were completely distinguishable. Particles coated in DiI alone labelled cells red, others coated with DiO labelled cells green and those particles coated with both DiI and DiO labelled cells yellow. Multicolour labelling made it possible to distinguish the fine processes and nuclei of adjacent cells from one another. The labelling process does not appear to cause major disruption of cell function since RGC dendrites and growth cones were seen to undergo dynamic changes in shape (Figure 5.4a) similar to those observed using other techniques (O’Rourke et al., 1994; Zheng, 2000). The process of INM continued in an apparently normal manner for several hours following biolistic labelling. Long periods of illumination of fluorescently labelled cells with laser light can result in phototoxic damage. In order to minimise these effects, laser intensity and the frequency of image acquisition were reduced to the minimum necessary to obtain clear images and adequate resolution of the movement of cells. At acquisition frequencies of ~0.001Hz and low laser powers preparations could
be imaged for several hours without obvious damage. The limiting factor in such recordings was usually the physical stability of the preparation rather than its viability. Cells labelled using the biolistic technique were of similar morphology to those seen in retinal whole-mounts labelled by placing DiI crystals on the ventricular surface. DiI crystals labelled large numbers of cells whose morphology was the same as those labelled with the gene-gun. This suggests that bombardment with biolistic particles does not do damage to cells over and above any that may be caused by DiI itself. Cells labelled using crystals showed INM but the density of the labelling was such that it was virtually impossible to distinguish between individual cell’s somas or their processes.

Cells biolistically labelled with DiI and co-labelled with Ca^{2+} dyes showed patterns of [Ca^{2+}]_i activity similar to those seen in cells labelled with Ca^{2+} dye alone. Co-labelling was achieved by loading retinas with Fluo-4 by bath immersion followed by biolistic labelling with DiI. Figure 5.4b shows spontaneous [Ca^{2+}]_i activity in a cell labelled with Fluo-4 alone and in another in the same preparation that was additionally labelled with DiI. Both cells show spontaneous [Ca^{2+}]_i transients like those seen in other preparations loaded with Ca^{2+} indicator alone.

5.3.2 The morphology of migrating retinal cells as identified by biolistic labelling

Over 50 E5 retinal whole-mounts were labelled with DiI and DiO and more than 300 cells imaged for the purposes of the experiments described in this chapter. A migrating cell typically had a cell body that was 18.6±0.8μm long and 6.0±0.4μm in diameter (n=50), and a characteristic bipolar shape (Figure 5.2a) with processes that extended to the VZ and GCL. The ventricular process was usually, but not always, thicker than the vitreal process. The size and shape of these migrating cells is similar to that of PCs described in vivo and in vitro (e.g. Sidman et al., 1959; Chenn and McConnell, 1995; Fischer et al., 2002). Thus, it was not possible to distinguish between PCs and NDCs on the basis of their morphology when they were moving away from the VZ. Since NDCs are born in the VZ and migrate away to other levels in the retina it can be assumed that all cells moving towards the VZ are PCs in G_2 of the cell cycle, whilst the population of
cells moving away from the VZ, contains both PCs in G1 and NDCs. Since Dil labelling only poorly survives fixation and membrane permeabilisation (for discussion see Molecular Probes Catalogue), and given the technical difficulty of relocating individual cells on the confocal microscope following immunolabelling, no attempt was made at this time to determine the identity of the cells moving away from the VZ by, for example, immunocytochemical staining for TuJ-1 or a marker for PCs.

The cell shown in Figure 5.5 was imaged at 0.001Hz over a period of 75mins and is typical of the cells (PCs) moving towards the VZ. At the start of the recording, the cell’s nucleus was located in the central retina. During the imaging period the cell nucleus, initially located 48μm away from the VZ, made saltatory movements (see below) towards it. The apical and basal processes were in contact with the VZ and the GCL at the outset of the recording. However, the basal process began to retract from the GCL after 15mins. The trailing process shortened as the nucleus moved towards the VZ, retaining its elongated shape throughout the first 45mins of the recording, before rounding up to form a sphere on its approach to the VZ. While elongated in shape, the speed of nuclear movement was ~30μm/h but this fell to ~2μm/h after the nucleus rounded up. This change in shape from elongate to spherical was seen for all cells as they approached the VZ. Thus, nuclei located between 20 and 80μm away from the VZ had an average length/width ratio of 3.2 (range 1.6-4.3), while those located in the VZ and up to 20μm away had an average length/width ratio of 1.1±0.1 (range 0.8-1.8).

The population of cells moving away from the VZ include both PCs and NDCs. The behaviour of the cell shown in Figure 5.8 is typical of the population of cells moving towards the GCL. At the start of the recording the nucleus of the cell shown in this figure was located in the ventricular half of the retina, some 50μm away from the VZ (Figure 5.6a). The image stacks shown were acquired at 0.001Hz. During the period of observation (75mins) the nucleus moved towards the GCL, in two periods of forward movement (at 30-45 and at 75mins, Figures 5.6b and d); the speed of movement during these periods was 12-17μm/h. The cell nucleus retained an elongated bipolar shape

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1 Since it is impossible to distinguish between PCs and NDCs moving away from the VZ, all descriptions of movements of cells migrating away from the VZ are termed INM for simplicity. However, it should be noted that these measurements are likely to include migrating NDCs.
throughout the whole recording (Figure 5.6c) and the apical process retained its contact with the VZ and the basal process with the vitreal surface. Thus, all cells imaged during movement towards the GCL behaved in a manner essentially identical to that seen for the nuclei of PCs undergoing INM toward the VZ.

5.3.3 Retraction of processes in preparation for mitosis

Previous descriptions of INM in cortical PCs (Chenn and McConnell, 1995) suggest that, prior to translocation of the nucleus, cells extend a process from the lumenal surface to the basal side of the VZ. Following the completion of G2 and prior to mitosis, cells are thought to retract their basal (vitreal) process. An example of a retinal cell retracting its vitreal process is shown in Figure 5.7a. This PC's nucleus moved from the GCL to the VZ covering a distance of approximately 20µm in 60mins before rounding up on its approach to the VZ. The vitreal process started to thicken and retract after the nucleus had begun to move, shortening by 24µm over a period of 40mins. Retraction of the ventricular process was only seen in a few cells. Since PCs are not thought to retract their ventricular processes during S-phase these cells may be NDCs migrating away from the VZ following terminal division. One of these cells is shown in Figure 5.7b. This cell's nucleus travelled 17µm toward the GCL over a 45min period. The ventricular process shortened and thickened and lost its contact with the ventricular surface, reducing its length by 18µm.

5.3.4 PCs undergoing INM move in a saltatory manner

The movements of the nucleus during INM are not linear, but instead are characterised by periods of forward movement interspersed with stationary periods and, occasionally, movement in the reverse direction. This is clear even in image stacks acquired at low frequencies. For example, the nucleus of the cell shown in Figure 5.8 undergoes three periods of movement towards the VZ during the period of observation (105mins). Between these bouts of movement, the nucleus is either stationary (as at 30mins) or moves only short distances (60mins) (Figure 5.8c). Such saltatory movements were seen in the majority of cells examined.
To examine the dynamics of INM in more detail, ‘virtual z sections’ were employed and images acquired at a frequency of 0.05Hz for short periods of time. At this more rapid sampling frequency (50 times faster than used for the observations described above), the movements of PC nuclei could be seen to be comprised of frequent short stationary phases that alternated with periods of movement in a forward or backward direction. Two examples of this movement are shown in Figure 5.9. During a period of 20mins the nucleus of cell 1 undergoes several periods of movement towards the VZ; the nucleus spent a total of 12mins making movements towards the VZ, interspersed with movements away from it (a total of 2min 40s) and periods during which the nucleus was stationary (a total of 3mins). The nucleus moved a total of 13µm toward the VZ in the 20mins period. By contrast, fewer forward movements were made by the nucleus of cell 2. This nucleus exhibited a total of 10min moving toward the VZ, 4mins 40s moving away from it and was stationary for 5mins 20s. It moved only 5µm towards the VZ during this time. The average distance traversed by the fast-moving cell (1) during each 20s period of forward movement was $1.4±0.7\mu m$ (n=18 periods of 20s) while the distance traversed by the slower moving cell was $0.3±0.1\mu m$ (n=15). The faster moving cell showed more periods of forward movement and travelled further with each jump than the slower moving cell. Thus, the overall speed of INM depends on the duration of the forward and backward movements, the time spent in the stationary state and the speed of the movement.

5.3.5 Speed of nuclear translocation during INM

Although there were considerable variations in the speed of movement of individual cells, the average speed of INM in whole-mount E5 retinae was $20.2±1.6\mu m/h$ (N=53, n=318). The average speed of movement of nuclei moving towards the VZ (i.e. PCs in G2) was $19.5±1.2\mu m/h$ (range 6.8-68.6µm/h; n=154), whilst those moving away from the VZ did so at an average speed of $20.8±1.9\mu m/h$ (range 4.8-57.1µm/h; n=164). The population of cells moving away from the VZ include both PCs in G1 and NDCs. Chenn and McConnell (1995) proposed that, in the cortex, these two populations migrate at different speeds, with immature neurons migrating at speeds ~10-fold greater than PCs. However, the distribution of speeds of migration of retinal PCs is very similar for cells moving towards and away from the VZ, with a single peak at 16-20µm/h (Figure 5.10).
There was no statistical difference between the mean speeds of INM in cells moving towards or away from the VZ (P=0.67, unpaired Student’s t-test performed on the transformed Lognormal distributions)

The speeds of INM were recorded between E5 and E7. In preparations imaged at 36°C, the average speed of INM at E5 was 20.2±1.6μm/h. There was little change at E6 (19.6±2.1μm/h; n=15) and a slight reduction in speed at E7 (16.3±2.3μm/h; n=6) although this was not statistically significant (P=0.26, unpaired Student’s t-test compared with E5). The rate of INM was temperature sensitive such that little or no movement was observed at RT (N=3). Increasing the temperature from 31°C to 35 & 36°C had little effect on the rate of movement. The average speeds of INM in E5 retinae were 17.5±3.5μm/h (n=9), 19.4±3.3μm/h (n=10) and 20.2±1.6μm/h (n=318) at 31, 35 and 36°C, respectively.

5.3.6 \([Ca^{2+}]_i\) transients are associated with movement

Spontaneous \([Ca^{2+}]_i\) transients occur in cells throughout the depth of the developing retina (see Chapter 3). The preliminary experiments described in this section examine whether or not spontaneous \([Ca^{2+}]_i\) transients of the kind described in Chapter 3 occur during INM and any role they may play in the movement of the nucleus. Attempts to measure \([Ca^{2+}]_i\) following labelling of cells with \(Ca^{2+}\) dyes using the biolistic dye-transfer technique proved problematic. Whilst cells were easy to label with \(Ca^{2+}\) indicators using this technique, it was difficult to control the amount of dye entering individual cells and the labelling was prone to rapid fading\(^2\). Given the problems encountered with biolistic labelling, retinae were instead loaded with Fluo-4 (10μM) by bath immersion and imaged as 'virtual z sections'. It is possible that the transient changes in Fluo-4 intensity seen in moving cells are due to movement rather than a change in \([Ca^{2+}]_i\). However, ratiometric measurements carried out using Indo-1 and Fluo-4, as described in Chapter 2, demonstrated that the changes in fluorescence intensity detected with Fluo-4 accurately reflect changes in \([Ca^{2+}]_i\) (see Figure 2.2 in Chapter 2). Since Fluo-4 is more resistant to fading than Indo-1, Fluo-4 alone was used

\(^2\) This problem was also encountered with \(Ca^{2+}\) indicators other than Fluo-4, including Oregon Green BAPTA-2, Calcium Green-2 and Indo-1.
for the experiments described here. Dye-fade and movement of the preparation limited the length and success of these recordings but INM was successfully followed in 10 cells. As shown in Figure 5.11, these cells’ nuclei moved in a manner similar to that observed in cells labelled with DiI using the biolistic technique; the nuclei moved in a series of small jumps in either a forward or a backward direction. The average speed of INM in the 10 cells imaged was 23\mu m/h (n=10), which is comparable with the rates of INM measured using DiI labelling (see above). Thus, INM does not appear to be adversely affected by labelling with Fluo-4. Figure 5.12 shows that transient changes in [Ca^{2+}], often occurred immediately prior to a period of movement. The rates of INM in the 2mins following a [Ca^{2+}], transient ranged from 16-46\mu m/h. Buffering of changes in [Ca^{2+}], by incubation with 50\mu M BAPTA-AM greatly reduced spontaneous [Ca^{2+}], activity and caused a decrease in the speed of migration (7\pm2\mu m/h, N=2, n=10, P<0.01, paired Student’s t-test), compared to 19\pm2\mu m/h in controls (N=2, n=10). Removing Ca^{2+} from the extracellular solution also has a marked effect, reducing the rate of migration to <5\mu m/h (N=2, n=8). However, the removal of extracellular Ca^{2+} caused significant changes in the morphology of cells and it was not possible to accurately assess the effect on the speed of migration. The preliminary findings described above suggest that spontaneous [Ca^{2+}], transients precede movements of the nucleus and are important in the process of INM.

5.3.7 The effects of neurotransmitters on INM in the E5 chick retina

The experiments described in Chapter 4 have shown that stimulation and blockade of muscarinic and purinergic receptors can affect the speed at which cells progress through mitosis. Exposure of the embryo to agonists and antagonists of these two receptors led to changes in the level of cell proliferation in the developing retina, results that suggest that ATP and ACh affect the cell cycle in the longer term. ATP and ACh, as well as affecting the rate of mitosis, could further affect the rate of the cell cycle through regulating the time it takes for a PC to undergo INM, in either or both of G1 and G2 of the cell cycle. Ca^{2+} imaging experiments were performed to determine whether or not neurotransmitter-evoked Ca^{2+} responses in cells with nuclei located outside the VZ have any effect on the rate of INM. Retinae were loaded with Fluo-4 and the response of cells monitored in z-line scans at 0.25Hz. UTP (10\mu M) and CCh (50\mu M) induced changes in
[Ca\(^{2+}\)], in a large number of cells throughout the depth of the retina, including cells with their nuclei located in the VZ, central retina and prospective GCL (Figures 5.13 and 5.14). GABA and glutamate (both 20\(\mu\)M) evoked responses from smaller numbers of cells throughout the depth of the retina but with the response strongest within the immature GCL (data not shown).

In order to examine the effects of neurotransmitters on cell movement the biolistic labelling technique described above was used to label cells with Dil and preparations were imaged at a frequency of 0.001 Hz for 1-3 hours. The average speed of cell movements including both cells moving towards, and cells moving away from, the VZ was significantly decreased by UTP (10\(\mu\)M) (17±1\(\mu\)m/h; N=6, n=59) compared to paired controls (22±1\(\mu\)m/h; n=48; P=0.01, paired Student's t-test), and significantly increased in the presence of the purinergic antagonist PPADS (30\(\mu\)M) (22±1\(\mu\)m/h; N=6, n=55 in PPADS compared to 186±1\(\mu\)m/h; n=77 in controls; P<0.01). CCh (10\(\mu\)M) caused an increase (19±1\(\mu\)m/h; N=3, n=82 in CCh, 17±1\(\mu\)m/h, n=23 in control, P=0.14), and pirenzipine (25\(\mu\)M) a decrease (18±1\(\mu\)m/h; N=3, n=36 in pirenzipine, 21±1\(\mu\)m/h; n=26 in control, P=0.09) in the speed of movement, although these effects were not significant. GABA, glutamate (both 20\(\mu\)M), bicuculline and NBQX (both 25\(\mu\)M) are without effect on INM (N. Luneborg, personal communication). Neither purinergic nor muscarinic drugs had any effect on the rate of movement in PCs moving towards the VZ (Figures 5.15 and 5.16). Cells moving towards the VZ in the presence of UTP moved at a rate of 18±1\(\mu\)m/h (N=6, n=43, P=0.21, paired Student’s t-test) compared to 21±2\(\mu\)m/h (n=21) in controls. Similarly, muscarinic stimulation was without effect; cells moved towards the VZ at speeds of 19±1\(\mu\)m/h (N=3, n=42, P=0.45) in the presence of CCh and 17±2\(\mu\)m/h (n=17) in controls. However, purinergic and muscarinic stimulation and blockade did have some statistically significant effects on the migration of cells moving away from the VZ (Figures 5.15 and 5.16). Cells moved away from the VZ at 17±2\(\mu\)m/h (N=6, n=16, P=0.03) in the presence of UTP compared with 23±2\(\mu\)m/h (n=27) in controls. Conversely, the rate of movement away from the VZ was slightly faster in the presence of PPADS (24±2\(\mu\)m/h, N=6, n=36, P<0.01) than in the respective control group (18±1\(\mu\)m/h, n=47). Muscarinic stimulation had no significant effect on the rate of movement away from the VZ (CCh 19±1\(\mu\)m/h, N=3, n=40, P=0.21 compared with
$17\pm2\mu m/h \ n=6 \ in \ controls)$. The muscarinic inhibitor, pirenzipine, did cause a slight reduction in the rate of movement compared with controls ($pirenzipine \ 18\pm2\mu m/h, \ N=3, \ n=12, \ P<0.05$ and control $24\pm2\mu m/h, \ n=15$). The population of cells moving away from the VZ consist of both PCs and NDCs. Since these two populations cannot be distinguished on the basis of their morphology alone, it was not possible to determine whether or not these drugs affected cell movements in one or both types of cell.
5.4 Discussion

5.4.1 The ‘DiOlistic’ technique and INM in the chick retina

Gan et al.'s (2001) ‘DiOlistic’ technique described in this chapter enabled the rapid labelling of individual cells in living retinæ with carbocyanine dyes. This technique has several advantages over other methods employed for the vital labelling of cells. Application of Dil crystals to the retina labels large numbers of closely apposed PCs, making it almost impossible to distinguish individual profiles. Biolistic transfection of cells with GFP has proved a useful technique for studying individual progenitors neurons in brain slices (e.g. Noctor et al., 2001). However, expression of GFP requires a delay of several hours. Biolistic application of lipophilic dyes allows cells to be imaged within minutes of application, a distinct advantage in physiological studies where the tissue may deteriorate with time. The use of combinations of carbocyanine dyes can label adjacent cells in different colours making it easier to distinguish one cell from another. The “DiOlistic” technique described in this chapter was effective at labelling the full extent of both PCs and RGCs and had no apparent detrimental effects, provided exposure to illumination by the laser was kept within reasonable levels. Further, retinæ can be labelled with Fluo-4 AM prior to labelling with DiI using the biolistic technique. This combined technique may be useful in future studies where measurements of $[Ca^{2+}]_i$ also require the detailed resolution of a cell’s processes in intact tissue.

Much of the work investigating the processes of cell migration in the nervous system has been carried out in the cortex, focusing on immature or adult neurons. Very little is known about cellular movement in PCs. Multicolour “DiOlistic” labelling of individual PCs in conjunction with confocal microscopy offers the opportunity to study the basic mechanisms of PC INM in real time in the developing chick retina. Sauer (1935) proposed that PCs of the neural tube migrated back and forth across the proliferative zone, such that cells were at the ventricular surface during mitosis but at deeper regions of the proliferative zone at other stages in the cell cycle. Experiments using the tracer $^3$H-thymidine (Sidman et al., 1951) confirmed that DNA synthesis occurs at the opposite side of the proliferative zone to mitosis. As predicted from anatomical studies (Seymour and Berry; 1975, Nagele and Lee, 1979), the investigations presented in this chapter show that retinal PCs undergoing INM have a characteristic bipolar morphology with an
elongated cell body. PC processes project to either side of the retina and the nucleus translocates within the processes either towards or away from the VZ. The cell rounds up upon approach to the VZ and the vitreal process is then retracted in preparation for mitosis. A small population of cells moving away from the VZ, which are presumably NDCs, were observed retracting their ventricular processes. Biolistic labelling alone does not permit the distinction of PCs from NDCs, although in theory this could be achieved by post-hoc immunostaining for neuronal or PC markers. However, since mitosis only occurs at the VZ, all cells showing movement towards the VZ are likely to be PCs, whilst the population of cells moving away from the VZ consists of both PCs and NDCs. PC movement is characterised by the frequent alternation of periods of forward movement with phases in which the nucleus is stationary or moves at a much reduced rate. Cells moving away from the VZ showed similar saltatory patterns of movement. This saltatory mode of movement has also been observed in migrating immature neurons in acute cerebellar (Komuro and Rakic, 1995) and cortical (Nadarajah et al., 2001) slice preparations.

The average speed of INM in the retina was 20µm/h and was similar for cells moving towards and away from the VZ. The velocity of these movements lies within the range of speeds recorded for neuronal migration. In the developing cerebellum, granule cells move at speeds ranging from 4 to 15µm/h, with region-specific differences (Komuro and Rakic, 1998; Komuro et al., 2001) and in the cortex, cells have been reported to move at speeds ranging from 13 to 60µm/h (O'Rourke et al., 1992, 1997; Takahashi et al., 1996; Nadarajah et al., 2001). The speeds of migration recorded here in the chick retina are very similar to those of PCs in the VZ of the ferret cortex (~18µm/h; Chenn and McConnell, 1995). Chenn and McConnell proposed that PCs and immature neurons move away from the VZ at different speeds. They found that the two daughter cells resulting from a vertical division (a proposed anatomical correlate of a ‘symmetric’ division producing two PCs) division moved away from the VZ at a similar rate, and at a speed much slower than that seen during G2 when cells are moving towards the VZ. Conversely, when cells divided horizontally (an ‘asymmetric’ division producing an NDC and a PC) the apically located PC (i.e. the daughter nearest the cortical surface) moved away very slowly (~1µm/h), whilst the basal daughter (an NDC) moved at a rate 10-fold faster. However, in the retina the average rates of migration both away from and
toward the VZ were virtually the same. Whilst the proportion of proliferative divisions in the retina is high at E5, a large number of neurons are born at this time (as demonstrated by the presence of TuJ-1 staining in the VZ; see also Prada et al., 1991; Snow and Robson, 1994) and so the measurements taken here for cells moving away from the VZ are likely to include a significant number of NDCs. However, despite measuring the velocities of 164 cells moving away from the VZ no indication was found of a population of cells moving towards the GCL at speeds significantly greater than another. Further, a wide-range of velocities of INM was observed for both the PC-only (i.e. towards the VZ), and the mixed population of PCs and NDCs, in the retina. Recent evidence has questioned whether horizontal divisions are necessarily neurogenic (Silva et al., 2002, see Chapter 4 for further discussion). Thus, the fast moving cells observed by Chenn and McConnell may not be a separate population comprised of immature neurons but rather simply represent an extreme in the distribution of velocities.

Assuming the average speeds of INM measured in this chapter, a cell would make the ~120μm journey from the VZ to the GCL and back in 5-6h. Mitosis takes ~30mins at E5 (see Chapter 4). These estimates fit well in the cell cycle time in the early embryonic chick retina as measured by others (6-10h; Morris and Cowan, 1995). The duration of the cell cycle increases throughout development; the chick retinal cell cycle increases from 6.8h to 10.1h over the proliferative period and extends to 16h at the end of cell generation (Morris and Cowan, 1995). Similar increases are seen in the mouse cortex, where PC cell-cycle length rises from a low of 8h to a peak of over 20h (Takahashi et al., 1995). However, this lengthening was not reflected in a statistically significant reduction in the velocity of INM in the chick retina, at least between E5 and E7. This may be explained if cells come to a halt at some point in the cell cycle and remain stationary for longer periods at later times in development. The study described here only considers cells that were in motion during the imaging period.

In the cortex, clonal analysis studies have revealed that whilst the majority of the members of a clone remain within a spatially restricted region (Cai et al., 1997b), others are located at significant distances away from the main group (Walsh and Cepko, 1993; Mione et al., 1994). Recent investigations have revealed that post-mitotic cortical and retinal neurons can undergo both radial and tangential dispersion (Fekete et al., 1994;
O'Rourke et al., 1997; Komuro et al., 2001). There has been some debate regarding whether PCs can also undergo tangential dispersion. Reid et al. (1995) proposed that PCs moved through the cortical VZ, pausing periodically to generate subclones. However, live imaging of PCs in the ferret cortex showed that these cells moved only radially and not tangentially (Chenn and McConnell, 1995). Similarly, in the E5 chick retina all observed PC movements were in the radial plane. Cells moved up and down between the VZ and the GCL and tangential movements were absent, an observation that suggests that tangential dispersion occurs after NDCs reach the layer appropriate to their fate. This hypothesis is supported by studies in a transgenic mosaic mouse retina (Reese and Tan, 1998). In these studies, retinal PCs migrated exclusively radially and tangential migration was found to be a property of postmitotic rather than proliferative cells.

5.4.2 $[Ca^{2+}]_i$ transients are temporally correlated with INM

It is possible that $[Ca^{2+}]_i$ transients may drive nuclear translocation during INM, via mechanisms similar to those driving neuronal migration elsewhere in the CNS. Previous studies have shown that transient elevations in $[Ca^{2+}]_i$ are essential for initiating and maintaining movement in a variety of cell types. The migration of immature cerebellar granule cells is significantly impaired by the removal of extracellular $Ca^{2+}$ or by the depletion of intracellular $Ca^{2+}$ stores (Komuro and Rakic, 1996). In the same study, $[Ca^{2+}]_i$ transients were shown to be temporally correlated with periods of forward movement (Komuro and Rakic, 1996). A similar correlation is described here, for cells in the chick retina. The frequency of $[Ca^{2+}]_i$ transients observed in retinal PCs (~12/h) is similar to that seen in migrating postmitotic neurons in the cerebellum (~13/h; Komuro and Rakic, 1996). Further, spontaneous $[Ca^{2+}]_i$ transients appeared to be temporally coordinated with periods of movement during retinal PC INM and occurred immediately prior to a period of nuclear translocation. The limited number of successful experiments carried out prevents any strong conclusions from being drawn. However, in these preliminary experiments chelation of $[Ca^{2+}]_i$ and removal of extracellular $Ca^{2+}$ caused significant reductions in the speed of INM. Dye fading severely limits the period over which $[Ca^{2+}]_i$ transients associated with cell movements could be followed using the techniques developed for the experiments described in this chapter. This problem might be circumvented by the use of more fade-resistant $Ca^{2+}$ dyes. Further assessment of the
role of \([\text{Ca}^{2+}]_i\) transients in driving INM will require the use of a technique that enables
\([\text{Ca}^{2+}]_i\) changes to be followed for longer periods of time.

Exactly how \([\text{Ca}^{2+}]_i\) transients exert control over cell migration remains unclear. The
similarities between the \([\text{Ca}^{2+}]_i\) changes seen in migrating immature neurons in the
brain, and during INM in retinal PCs, suggest that a similar mechanism may be involved
in the movement of both progenitor nuclei and neuronal cell bodies. During neuronal
migration, the cell moves via mechanisms that involve microtubules and their associated
motor proteins (Rakic et al., 1996). \([\text{Ca}^{2+}]_i\) transients may regulate the dynamic
assembly and disassembly of these cytoskeletal elements. For example, the platelet-
derived growth factor-stimulated migration of cultured oligodendrocytic PCs was
inhibited both by chelation of \([\text{Ca}^{2+}]_i\) and by inhibition of either actin or tubulin
polymerisation (Simpson and Armstrong, 1999).

Neurotransmitters are intimately involved in the regulation of neuronal migration
(Komuro and Rakic, 1992, 1993, 1996; Rossi and Slater, 1993). Glutamate, acting via
the NMDA receptor, positively modulates the rate of cerebellar granule cell migration,
whilst GABA is without effect (Komuro and Rakic, 1993). The results presented in
Chapters 3 and 4 show that purinergic and muscarinic receptors are involved in the
generation of the spontaneous \([\text{Ca}^{2+}]_i\) transients seen in VZ cells, and that both
stimulation and blockade of these receptors affect the rate of mitosis. Cells throughout
the depth of the retina respond to purinergic and muscarinic stimulation with a change in
\([\text{Ca}^{2+}]_i\). However, neither muscarinic nor purinergic drugs had an effect on the rate of
INM in the population of PCs moving towards the VZ (i.e. in \(G_2\)). UTP significantly
slowed INM in the population of cells moving away from the VZ while CCh speeded it
up, although this was not a statistically significant effect. PCs and NDCs moving away
from the VZ cannot be distinguished on the basis of their morphology alone. Thus, it
was not possible to determine whether or not these drugs affected cell movements in one
or both cell types. Further, whilst these effects were statistically significant when tested
against the respective paired controls, the average speeds of migration in all test and
control groups were very similar. All experimental and control groups consisted of data
spanning a similarly wide range of speeds.
Given the profound effects of both purinergic and muscarinic drugs on \([\text{Ca}^{2+}]_i\), it is perhaps surprising that they should have only minor effects on cell migration. Further experiments will be necessary to determine why this is so.

5.5 **Further studies**

Further work is required to develop an imaging technique that allows high temporal resolution of \([\text{Ca}^{2+}]_i\) transients and their measurement in cells over an extended period. The preliminary experiments described here suggest that \([\text{Ca}^{2+}]_i\) transients may be important in the translocation of the nucleus during INM. However, further experiments are required to establish the precise role of \([\text{Ca}^{2+}]_i\) signals in this process. The lack of effect of transmitters that have profound effects on \([\text{Ca}^{2+}]_i\) on INM is surprising and requires further investigation.

5.6 **Summary**

During development, the nuclei of retinal PCs undergo INM, moving from the VZ to the prospective GCL in G1 of the cell cycle, before duplicating their DNA in S-phase, and returning to the VZ in G2 and undergoing mitosis. Work presented in chapters 3 and 4 has shown that purinergic and muscarinic receptors are involved in generating spontaneous \([\text{Ca}^{2+}]_i\) transients amongst VZ cells, and that both stimulation and blockade of these receptors affect the rate of mitosis. The experiments in this chapter tested whether or not muscarinic and purinergic, along with glutamatergic and GABAergic, receptors further affect the rate of the cell cycle through regulating the time a cell takes for INM. Surprisingly, muscarinic and purinergic drugs had little effect on the rate of PC INM. Similarly, but consistent with their lack of effect on mitosis, neither GABA nor glutamate affected INM. The developmental significance of the lack of effect of these neurotransmitter systems is, at present, unclear.

The preceding chapters have examined the ability of four neurotransmitter systems to modulate both \([\text{Ca}^{2+}]_i\), and different aspect of the proliferative cell cycle prior to synaptogenesis. There is increasing evidence to indicate that the RPE performs an important regulatory role in the development of the neural retina (see Chapter 4). In the following chapter, the possibility is examined that an alternative form of non-synaptic communication, that of gap-junctional coupling, may provide a pathway by which the RPE can influence the neural retina.
INM during the PC proliferative cycle. Following mitosis, proliferating cells extend a process from the VZ to the GCL prior to translocation of the nucleus to the vitreal surface in $G_1$ of the cell cycle. The nucleus returns to the VZ in $G_2$ of the cell cycle and the vitreal process retracts prior to mitosis. The term INM describes the process of nuclear translocation during $G_1$ and $G_2$. NDCs migrate from the VZ following a terminal division. Thus, cells moving towards the VZ are PCs in $G_2$, whilst those leaving the VZ may be PCs in $G_1$ or NDCs.
Figure 5.2 ‘DiOlistic’ labelling of retinal cells. A, an xz projection of a two colour image stack (100 images, 1 μm steps) of a PC, the nucleus of which moved toward the VZ. The cell has been labelled with a combination of Dil and DiO using the “DiOlistic” technique (see next figure for details). Note the thicker nature of the process extending toward the VZ Process. B, an xy projection of 20 images (1 μm steps) through the thickness of an E7 RGC labeled with Dil. Scale bars 10 μm.
Figure 5.3. A combination of Dil and DiO allows neighbouring cells to be labelled different colours and more easily distinguished from one another. A, a projection of a two colour image stack (85 sections, 1μm steps) of cells from an E5 retina labelled with both Dil and DiO. The 568nm and 488nm lines of the HeNe and argon lasers were used to excite fluorescence of Dil and DiO, respectively, and the emitted light was collected at 590nm and 522nm. Particles coated in Dil alone labelled cells red (arrow head) whilst others coated with DiO alone labelled cells green (thick arrow). Particles coated with both Dil and DiO labelled cells yellow (see example in previous figure). B, The same region collecting the Dil signal only. C, The same region collecting the DiO signal only. Scale bar 10μm.
Figure 5.4. Biolistic labelling does not disrupt dynamic and physiological changes in retinal cells. A, a time-lapse series of images of an RGC labelled with DiO undergoing axon extension. Images are xy projections of image stacks (10 images, 1μm step interval). The tip moves more than 10μm in the 7.5mins shown here. Scale bar 10μm. B, Spontaneous [Ca^{2+}] transient occurring in cells biolistically labelled with Dil are the same as those seen in controls. Images show xy images of a region of retina loaded with Fluo-4 AM and then shot with Dil. Left, shows the fluorescence collected from Dil alone, centre, shows the Fluo-4 emission from the same region during a spontaneous [Ca^{2+}] transient occurring in a DiI+Fluo-4 labelled cell (thin arrow) and right, shows the Fluo-4 emission from the same region taken during a spontaneous [Ca^{2+}] transient occurring in an adjacent cell, labelled with Fluo-4 alone (open arrow). Scale bar 5μm. Traces show the spontaneous [Ca^{2+}] events occurring in the two cells indicated in the panel above. Arrows on traces indicate the time at which the images in B (centre and right) were taken.
Figure 5.5. INM in PCs moving towards the VZ. A, a time-lapse series of image stacks (85 images, 1μm steps) of a PC labelled with DiO using the “DiOlistic” technique, in an E5 retina. An asterisk show the original position of the nucleus, an arrow indicates the current position of the nucleus in each image and an arrow head indicates the terminus of the vitreal process. The cell body rounds up on approach to the VZ. Scale bar 10μm. The distance travelled (B), the length/width ratio of the cell body (C) and the speed of movement (D) are each shown as a function of time.
Figure 5.6. INM in cells moving towards the GCL. A, a time-lapse series of image stacks (100 images, 1μm steps) of a cell labelled with DiO using the "DiOlistic" technique, in an E5 retina. The cell nucleus makes saltatory movements towards the GCL. An asterisk denotes original position of the nucleus and an arrow indicates the current position of the nucleus in each image. Scale bar 10μm. The distance travelled (B), length/width ratio of the cell body (C) and speed of movement (D) are shown as a function of time. The cell body remains elongated throughout the period imaged.
Figure 5.7. Process retraction in PC and NDCs in E5 retinae. A, Time-lapse series of a PC labelled with Dil moving from the GCL to the VZ. The process extending to the vitreal surface retracts by 18μm over the 60min imaged period. B, A Dil-labelled NDC moves towards the GCL and retracts its ventricular process. Images are projections of image stacks (105 and 75 images, respectively, 1μm steps) of cells labelled with Dil using the “DiOlistic” technique. Scale bars 10μm.
Figure 5.8. INM is saltatory in PCs moving towards the VZ. A, a time-lapse series of xz projections of image stacks (95 images, 1μm steps) of a PC, the nucleus of which moved towards the VZ in jumps. The PC has been labelled with Dil using the “DiOlistic” technique. An asterisk denotes the original position of the nucleus and an arrow indicates the current position of the nucleus in each image. Scale bar 10μm. B, The distance travelled by the cell soma as a function of time. C, Speed of movement at each time point.
Figure 5.9. INM at higher image acquisition frequencies. The movements of two example PCs are shown. A, Cell 1 moves rapidly towards the VZ (average 39µm/h) whilst B, Cell 2 moves more slowly (12µm/h). The top traces in A and B show the distance travelled by the cell nucleus as a function of time. The direction (µm) moved by each cell in a 40s period is shown in the histograms below each trace. Positive values represent movement towards the VZ, whilst negative values represent a movement towards the GCL. Note the different scales on the y axis of Cell 1 and Cell 2.
Figure 5.10. Speeds of cell movement in PCs moving towards the VZ (top) and PCs and NDCs moving away from the VZ (bottom) in E5 retinae. The distributions of speeds were similar for both populations. The individual rates were sorted into 5 μm/h bins. Data comes from 53 retinae (cells moving towards the VZ, n=154; cells moving away from the VZ, n=164).
Figure 5.11. [Ca^{2+}]_transients are temporally correlated with cell movement in migrating retinal PCs. A, Single confocal images of a ‘virtual z-section’ through an E5 retinae loaded with Fluo-4 showing changes in [Ca^{2+}]_ in a migrating PC. The images have been pseudo-coloured and warm colours indicate increasing [Ca^{2+}]. B, Trace showing changes in [Ca^{2+}]_ in the soma of the cell shown in (A) as ΔF/F. Three transients occurred during the imaged period and these are indicated with red arrows. C, Plot of distance of the soma from the VZ as a function of time. D, The direction and rate of movement during 40s periods across a 20min recording. Note that the first transient is correlated with a backward movement whilst the other two correlate with periods of forward movement. The largest transient occurred just prior to the fastest period of movement.
Figure 5.12. Relationship between $[\text{Ca}^{2+}]$, transients and PC INM. $[\text{Ca}^{2+}]$ transients are temporally correlated with cell movement in migrating retinal PCs. A, (i-iii) Temporal correlation between the distance moved towards the VZ (i), changes in $[\text{Ca}^{2+}]$, as shown by increasing $\Delta F/F$ (ii) and the speed of movement at each time point (iii). All data is from a single E5 PC moving towards the VZ, sampled at 40s intervals. B, (i-iii) A further example of the temporal correlation between movement and the occurrence of $[\text{Ca}^{2+}]$ transients, imaged at an increased image acquisition frequency. Data comes from a single PC moving toward the VZ in an E5 retina imaged at 10s intervals. Note the different scales on the y-axes. Changes in $\Delta F/F$ greater than 10% are indicated with arrows.
Figure 5.13. UTP evokes [Ca^{2+}]_i responses in cells throughout the depth of the retina. Example images from a z-line scan (x1 average) time-lapse movie, in which UTP (50μM) was applied to an E5 retina labelled with Fluo-4 AM. Scale bar 10μm.
Figure 5.14. CCh evokes $[Ca^{2+}]_i$ responses in cells throughout the depth of the retina. Example images from a z-line scan (x1 average) time-lapse movie, in which CCh (50μM) was applied to an E5 retina labelled with Fluo-4 AM. Scale bars 10μm.
Figure 5.15. The effects of purinergic receptor activation and blockade on INM. Graphs show the effects of (A) UTP (10μM; N=6) and (B) PPADS (30μM; N=6) on the speed of INM in all cells (left), in PCs moving towards the VZ (middle) and in PCs and NDCs moving away from the VZ (right). All data come from E5 retinae. Values are means±SEM, *P<0.05, **P<0.01, paired Student’s t-test.
Figure 5.16. The effects of muscarinic receptor activation and blockade on INM. Graphs show the effects of (A) CCh (10μM; N=3) and (B) pirenzipine (25μM; N=3) on the speed of INM in all cells (left), in PCs moving towards the VZ (middle) and in PCs and NDCs moving away from the VZ (right). All data come from E5 retinae. Values are mean±SEM, *P<0.05, **P<0.01, paired Student's t-test.
Chapter 6

Tracer coupling and Ca\(^{2+}\) signalling within and between the RPE and the VZ during early development of the chick retina

6.1 Introduction

Coupling between cells in the developing cortex and retina

Co-ordinated spontaneous [Ca\(^{2+}\)]\(_j\) transients occurring within neighbouring cells have been observed in the embryonic cortical VZ (Owens and Kriegstein, 1998). These domains of localised [Ca\(^{2+}\)]\(_j\) activity are triggered by one or two cells and involve groups of up to 20 of their neighbours. Owens and Kriegstein propose that these [Ca\(^{2+}\)]\(_j\) signals propagate between groups of PCs via gap junctions and may serve to synchronize cell cycle events. In the developing postnatal cortex, co-ordinated spontaneous [Ca\(^{2+}\)]\(_j\) transients, or 'domains', occur in columnar patches (Yuste et al., 1995; Peinado et al., 1993b) and are believed to be spread via the transfer of 2\(^{nd}\) messengers, such as IP\(_3\) (Kandler and Katz, 1998), between gap junction-coupled neurons (Yuste et al., 1992, 1995; Peinado et al., 1993). It was postulated that this gap junction network represents a primitive cortical circuitry, prior to synapse formation. The role of gap junctional communication and Ca\(^{2+}\) signalling amongst cells of the embryonic retinal VZ has received little attention.

Gap junctions provide a major pathway for intercellular communication and electrical transmission in the developing and adult CNS. Extensive coupling has been recorded between neurons in both the developing rat cortex (Peinado et al., 1993) and the embryonic mammalian retina (Penn et al., 1994). Gap junction proteins are present in the retina at the earliest stages of development (Becker and Mobbs, 1999) and their expression is both temporally and spatially regulated (Fujisawa et al., 1976; Becker et al., 2002), suggesting a role for cell-cell communication in regulating proliferation and/or differentiation. In the embryonic chick eye, suppressing the expression of Cx43, one of the earliest gap junction proteins found in the retina, causes a reduction in proliferation, resulting in a small eye (Becker and Mobbs, 1999). Studies in the
mammalian neocortex have shown that PCs are coupled in columnar clusters that extend radially through the VZ (LoTurco and Kriegstein, 1991). It has been suggested that NDCs uncouple prior to their migration out of the cortical VZ (LoTurco and Kriegstein, 1991; Bittman et al., 1997), before forming new and distinct gap junctions with other postmitotic neurons in the developing cortical plate (Yuste et al., 1992; Peinado et al., 1993). This second wave of gap junction mediated communication is also apparently transient, declining with the onset of synaptogenesis (Peinado et al., 1993). Similar changes in the pattern of gap junctional communication have been observed in RGCs of the developing chick retina (Becker et al., 2002).

Influences of the RPE on proliferation

The RPE plays an important role in the normal development of the neural retina. When retinal cultures are allowed to develop in vitro, inclusion of RPE cells or RPE-conditioned media leads to a highly organized arrangement of differentiating cells similar to that seen in vivo (Vollmer et al., 1984; Sheedlo and Turner, 1996; Rothermel et al., 1997). In in vivo experiments in which mouse RPE cells were genetically ablated using a diphtheria toxin transgene, the animals presented eyes in which the laminar structure of the retina was completely disrupted (Raymond and Jackson, 1995). In albino animals, where the RPE lacks pigment, the neural retina undergoes abnormally high levels of proliferation followed by enhanced cell death. The central neural retina remains under-developed and retinal axons undergo abnormal crossings at the optic chiasm leading to inappropriate innervation of the visual cortex (for review, see Jeffery, 1997). The nature of the signals between the RPE and the neural retina are unclear. Work on the albino retina suggests that dopa, a melanin precursor, can affect the cell cycle of dividing retinal cells. Exogenous application of dopa appears to normalise the patterns of cell production in albinos (Ilia and Jeffery, 1999), although the mechanisms by which this occurs are unclear. Thus, the presence of a pigmented RPE appears to play an important role in the ordered proliferation, differentiation and lamination of the neural retina.

Purinergic receptors have been found in bovine, rat and human RPE cells (Peterson et al., 1997; Ryan et al., 1999; Sullivan et al., 1997) and stimulation with either ATP or UTP cause changes in [Ca$^{2+}$], in cultured bovine RPE cells (Peterson et al., 1997).
Receptors for dopamine, ACh, adrenaline and adenosine (Friedman et al., 1988; Darry et al., 1990; Frambach et al., 1990) have also been identified in the adult RPE. However, little is known about neurotransmitter receptors in the RPE during early retinal development.

The experiments described in this chapter use Ca\(^{2+}\) indicators to examine the spread of spontaneous activity within, and between, the RPE and VZ, and to look for the presence of neurotransmitter-evoked [Ca\(^{2+}\)]\(_i\) signals in the chick RPE at E5. In addition, a gap junction-permeant tracer has been used to investigate the presence and nature of gap junctional coupling in and between the RPE and the neural retina of the developing chick retina.
6.2 Methods

The methods used in this chapter are described in detail in Chapter 2 (sections 2.4, 2.8 and 2.9). Briefly, spontaneous and evoked [Ca^{2+}]_{i} activity was investigated in whole-mount retinae loaded with Fluo-4 AM. In experiments to determine the effects of the presence of the RPE on [Ca^{2+}]_{i} activity in the neural retina, the central region of the retina was imaged with the GCL facing the objective. In this orientation the signal from the VZ is maximised since the photons emitted by the Ca^{2+} indicator do not have to pass through the RPE on their way to the photomultiplier. When examining the [Ca^{2+}]_{i} activity within the RPE, retinae were orientated with the RPE nearest the objective.

[Ca^{2+}]_{i} transients propagating between the RPE and the neural retina were assessed by imaging folded, Fluo-4 AM loaded, retinae in the xy plane to create 'virtual z sections' (see Chapter 2 for details), which included all layers of the neural retina and RPE. The speed of propagation of co-ordinated [Ca^{2+}]_{i} transients was determined by measuring the time taken for [Ca^{2+}]_{i} to increase >10% above baseline in cells located along a line perpendicular to the propagating wave front. The response of the RPE to neurotransmitters was assessed in preparations in which it had been carefully isolated from the neural retina. Preparations were kept at 36°C or RT when assessing spontaneous or neurotransmitter-evoked changes in [Ca^{2+}]_{i}, respectively.

Gap junction-mediated coupling was investigated by injecting cells with a combination of Neurobiotin and FITC-dextran using whole cell patch clamping. Injections were made either into RPE cells in E5 retinae in which the RPE was left in place, or in the end processes of PCs and NDCs and their cell bodies at the ventricular surface of E5 neural retinae in the absence of the RPE. Retinae were perfused with normal Krebs' solution and maintained at 36°C for 30mins after injection. To further confirm that Neurobiotin coupling was mediated via gap junctions, additional injections were carried out in the presence of the gap junction blocker carbenoxolone (100μM). Experiments involving the imaging or patch clamping of cells in the RPE required the removal of the overlying sclera. In order to minimise disruption of gap junctions between the RPE and the neural retina through mechanical stress, the RPE's attachment to the overlying sclera was weakened enzymatically by pre-incubation for 30mins with 0.05% dispase in 241
Krebs' solution, before careful dissection. Following injection, preparations were fixed and processed to render the Neurobiotin visible using streptavidin-Cy3. Some retinae were subsequently processed for neuron specific β-tubulin expression using a mouse monoclonal primary antibody (TuJ-1) and an anti-mouse Cy-5 tagged secondary antibody. The preparations were then examined on a confocal microscope (Leica or Zeiss, see Chapter 2). Unless otherwise stated, the images of dye coupling presented in this section are single xy confocal images or projection images viewed in the xz plane of a 3D stack. Stacks were created by taking xy confocal images at 1-2μm intervals throughout the depth of the retina. These xy images were built into a xyz 3D stack using Metamorph or Lucida software and rotated through 90° about the x axis and projected as a single image in the xz plane.

Staining for gap junction proteins was carried out following a protocol adapted from Becker et al. (1995). E5 frozen retinal sections (20μm thick) were exposed to primary antibodies against Cx43: Gap15 (rabbit polyclonal), and 1A (mouse monoclonal) (courtesy of DL Becker). Appropriate Alexa 488- or Cy3-tagged secondary antibodies were then used to enable fluorescent detection of Cx43. The sections were additionally treated with 5μM propidium iodide in PBS in order to stain cell nuclei.

Experimental groups were tested against their respective controls using an unpaired Student’s t-test, unless otherwise stated. Differences were considered statistically significant at one of two levels: * P<0.05 and ** P<0.01. The results are means ± SEM of each control or test group, where N=number of retinae investigated and n=number of cells recorded.
6.3 Results

I. Confocal imaging of Ca\(^{2+}\) signals in the RPE and the neural retina of the developing chick eye

Isolated spontaneous \([Ca^{2+}]_i\) transients occur in the majority of cells in the VZ of the E4-6 chick retina (Chapter 3). Co-ordinated spontaneous \([Ca^{2+}]_i\) transients occurring within neighbouring cells have been observed in the embryonic cortical VZ and are thought to propagate through gap junctions (Owens and Kriegstein, 1998). The \(Ca^{2+}\) imaging experiments presented in this section describe patterns of co-ordinated spontaneous \([Ca^{2+}]_i\) activity occurring in the RPE and neural retina of E4-6 chick eyes. Neurotransmitter-evoked changes in \([Ca^{2+}]_i\) occurring in the E5 RPE are also described.

6.3.1. Spontaneous \([Ca^{2+}]_i\) transients in the RPE

Spontaneous \([Ca^{2+}]_i\) activity is common in the developing RPE and takes one of two forms: \textit{transients} restricted to single cells and \textit{waves} which spread between neighbours. A wave was defined as an episode of activity that was spatially and temporally co-ordinated in a population of greater than 2 cells. Waves were seen throughout the RPE and at all stages examined (E4-6). The majority of waves spread radially, or occasionally asymmetrically, from a single trigger cell (Figure 6.1). At E5, the average speed of propagation away from the trigger cell was 7±1\(\mu\)m/s (range 1-17\(\mu\)m/s; data from 25 waves, \(N=4\) retinae). The number of cells involved in each wave varied widely (range 3-128), although events incorporated an average of 17±4 cells, covered an area of 500±95\(\mu\)m\(^2\) and lasted 16±1.8s (data from 95 waves, \(N=6\) retinae). Waves were seen at a frequency of 22.3±7.9 waves/500s/42x10\(^3\)\(\mu\)m\(^2\) (\(N=6\) E5 retinae). These waves occurred with an apparently random distribution. Occasionally, the same cells participated in more than one wave during the imaging period (see Figure 6.1a). The application of carbenoxolone (100\(\mu\)M) caused a significant reduction in the occurrence of these ‘wave’ events (1.7±0.8 waves/500s/42x10\(^3\)\(\mu\)m\(^2\); \(N=4\), \(P<0.01\) compared with control retinae) and the remaining activity involved only small numbers of cells. These

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1 42x10\(^3\)\(\mu\)m\(^2\) is the area of a field of view at a x40 magnification

243
results indicate that wave-like activity in the early RPE is mediated, at least in part, by gap junctional coupling. Single transients (see below) continued to occur throughout the application of carbenoxolone at a frequency similar to that seen in controls (82±3%, N=4, n=160 and 84±3%, N=4, n=180 respectively), a result that suggests that carbenoxolone does not affect the mechanisms that trigger \([Ca^{2+}]_i\) transients.

Spontaneous \([Ca^{2+}]_i\) transients occurring in single cells were widespread in the RPE between E4 and E6 (other times were not investigated) (Figure 6.2a). 84±3% of RPE cells examined (N=4, n=180) at E5 showed changes in \([Ca^{2+}]_i\) above criterion during the 500s recording period. The events had a mean duration at half-peak of 8.3±0.8s. The duration of these events was shorter and more uniform than those observed in the VZ (see Chapter 3). Unlike the neural retinal VZ, where the majority of active cells underwent only one or two such transients in 500s, RPE cells frequently underwent several changes in \([Ca^{2+}]_i\) in that time (Figure 6.2a & b) (mean 3.0±0.1 transients/500s, N=3, n=117). The interval between individual peaks varied. The frequency distribution of RPE \([Ca^{2+}]_i\) transients is shown in Figure 6.2b. At RT, the number of cells demonstrating spontaneous activity was reduced (by ~50%), and wave-like events were rare (2.1±0.8waves/500s/42x10^3\(\mu m^2\); N=3).

6.3.2. *Spontaneous co-ordinated \([Ca^{2+}]_i\) transients in the VZ in the absence of the RPE*

Spontaneous \([Ca^{2+}]_i\) fluctuations occurring in retinal VZ cells have already been described in Chapter 3; time-lapse confocal imaging of the VZ showed that \([Ca^{2+}]_i\) transients occur at low frequency, with cells undergoing ~1 event every 5mins. Some of these individual transients can also spread as waves, invading clusters of more than 35 cells (mean 17±2 cells; N=8 retinae). In these wave-like events the wave front usually spread radially to the surrounding cells and propagated between cells at an average speed of 11.4±2.0\(\mu m/s\) (n=25 waves, N=8 retinae) (Figure 6.4). Waves were seldom seen at E6 but were more common at E4 and E5; on average, 1.6±0.7 waves/500s/42x10^3\(\mu m^2\) were seen at E5 (N=8 retinae). The mitotic status of the cells involved in the waves was determined by comparison of the Hoechst and Fluo-4 movies. In a sample of 25 waves, ~80% of the cells within a wave boundary were in interphase and ~20% were in some stage of mitosis. Of these cells, the interphase cells...
usually all showed a co-ordinated rise in $[Ca^{2+}]$, while the mitotic cells did not (Figure 6.3 and 6.4). On the rare occasions that mitotic cells were involved in waves they were usually in prophase, and seldom in metaphase or anaphase. Thus, co-ordinated changes in $[Ca^{2+}]$ occur within small groups of adjacent cells in the VZ, but may exclude cells in the more advanced stages of mitosis. Waves on the scale of those seen in RGCs and amacrine cells in older retinae that involve hundreds of cells (Catsicas et al., 1998; Wong et al., 1998) were not seen.

The frequency of spontaneous $[Ca^{2+}]$ waves in the VZ in control solution was low but reliably observed. Co-ordinated activity was not seen in the presence of 100μM carbenoxolone (N=6, E5 retinae) but, following 30mins of washout of the drug, waves encompassing <15 cells were seen in 3 of the 6 preparations. This indicates that the wave-like events seen in the early embryonic neural retina are likely to spread through gap junctional coupling between cells. However, the low frequency of wave-like events prior to application of carbenoxolone and the only partial recovery of activity after washout make it difficult to draw definitive conclusions as to whether these events are mediated via gap junctions alone.

Imaging cells throughout the thickness of the retina, using ‘virtual z-scans’, showed that the wave-like events described above propagated through the depth of the retina as well as radially. Figure 6.5 shows an example of a spontaneous event, occurring in an E5 retina, which incorporates many cells throughout the depth of the retina. The average speed of propagation was 15±3μm/s (range 7-41μm/s; n=14). As when such waves were observed in the xy plane in the VZ, some cells within the boundary of the wave did not show any change in $[Ca^{2+}]$.

6.3.3. Spontaneous co-ordinated and isolated $[Ca^{2+}]$ transients in the VZ in the presence of the RPE

Spontaneous $[Ca^{2+}]$ activity in the neural retina could be affected by the removal of the RPE. In order to examine potential differences, retinae were imaged with the RPE intact and compared with ‘control’ eyes in which the RPE was removed. Ventricular cells demonstrated a higher level of spontaneous $[Ca^{2+}]$ activity when the RPE was in situ
than when it was removed. The proportion of cells undergoing spontaneous $[Ca^{2+}]_i$ activity in E5 retinae increased from 25±2% active/500s (N=7, n=372) in the absence of the RPE, to 65±4% (N=3, n=150, P<0.01) when the RPE was in place (Figure 6.6a). In any given cell, the duration and kinetics of the transients were the same as those seen in the absence of the RPE. However, the frequency of the transients occurring in cells increased in the presence of the RPE. In the absence of the RPE, cells that showed spontaneous $[Ca^{2+}]_i$ activity during the imaging period demonstrated 1.3±0.1 transients/500s (N=3, n=37), compared with 2.3±0.1 transients/500s (N=3, n=97, P<0.01) in the more intact preparation. The frequency distribution of spontaneous events is shown in Figure 6.6b. The reduction in both the number of spontaneously active cells and the frequency of events per cell in the absence of RPE could result either from damage or from the loss of some factor that stimulates the production of these transients following the removal of the RPE. Waves in the VZ, like those seen in the absence of the RPE, were seen when the RPE was intact. Again, these events invaded similar numbers of cells (~15) but occurred at a higher frequency than in the absence of the RPE (1.8 versus 0.7 events/500s; N=3 and N=6 respectively, P<0.01).

6.3.4. Transmission of spontaneous $[Ca^{2+}]_i$ transients between the RPE and the VZ

Experiments were carried out to investigate whether $[Ca^{2+}]_i$ transients propagate between the RPE and the VZ but this proved very difficult. One technique tried was to take repeated alternate xy scans at the level of the RPE and the VZ. However, the microscope could not step between the two levels at a rate fast enough to resolve any propagation between the two tissues. ‘Z-line’ scans permit a single line scan to be taken through the depth of the tissue, including both the RPE and the VZ. However, whilst the use of a ‘harp’ ensured that preparations remained stable in terms of any movement in the z-plane, small movements in the xy plane that occur when imaging over long periods caused a shift in the region of retina scanned and rendered the image sequences difficult to interpret. These small movements were sometimes present in the xy images used in the experiments described in preceding chapters but were readily compensated. To circumvent the problems described above, ‘virtual z-scans’ (i.e. images of folds in the RPE + neural retinal preparation) were used. However, the mechanical connection between the RPE and neural retina is very weak and the RPE frequently separated from
the retina reducing the number of successful ‘virtual z-scan’ experiments that could be done.

Despite the technical difficulty of these experiments a small number of examples of [Ca^{2+}]_i activity travelling between the RPE and the neural retina were obtained. Events that did pass between the two tissues included waves, involving many cells, and single transients that passed from individual RPE cells to single VZ cells. Activity was seen to pass both from the RPE to the VZ, and from the VZ to the RPE. Figure 6.7 shows an example of a [Ca^{2+}]_i wave travelling from the VZ to the RPE. This event incorporated many cells in both tissues. The wave was initiated in the neural retina and propagated through it at a rate of ~7µm/s. Approximately 60 neural retinal cells in the section imaged were involved in the event, and likely involved more at other focal planes. The wave propagated between the neural retina and RPE within the 3.1s interval between two successive scans. Transfer between the two tissues was therefore relatively rapid but due to the time resolution employed it was not possible to accurately determine the speed of propagation. Within the RPE the wave appeared to invade a large number of cells; again the scan frequency chosen prevented any estimate of the speed of propagation.

Figure 6.8 shows an example of a [Ca^{2+}]_i transient that crossed from the RPE to the VZ. A spontaneous rise in [Ca^{2+}]_i was seen in an RPE cell, that then crossed into the ventricular process of a cell in the neural retina before spreading along its length. The transient propagated along the length of the cell in the neural retina at a speed of ~9µm/s, although the maximal fluorescence change in the nucleus was not reached until ~25s after initiation.

Signals that propagated between the RPE and the VZ were only seen on 3 occasions in more than 30 ‘virtual z-scan’ preparations. It is possible that damage to the connections between the RPE and the VZ done during dissection reduced the frequency of these events. Further experiments are required to determine if this is so. Improved techniques in the isolation of neural retina+RPE preparations may reveal more extensive coupling between the two tissues and permit investigation of the mechanisms underlying these events. In any future investigations high image acquisition frequencies, combined with
more fade-resistant Ca\textsuperscript{2+} indicators, will need to be employed to properly resolve the transmission of waves between the VZ and the RPE and vice versa.

6.3.5. The embryonic RPE responds to muscarinic and purinergic stimulation with a change in \([\text{Ca}^{2+}]_i\).

Purinergic and muscarinic receptors have been located in the adult RPE along with those for dopamine, adrenaline and adenosine (Friedman et al., 1988; Dearry et al., 1990; Frambach et al., 1990; Petersons et al., 1997). RPE preparations were loaded with Fluo-4 and the underlying neural retina was carefully removed. This ensured that any changes in \([\text{Ca}^{2+}]_i\) resulted from direct stimulation of the pigment cells. CCh, ATP and UTP (all 100\mu M) produced marked increases in \([\text{Ca}^{2+}]_i\) in a significant proportion of RPE cells in the isolated E5 RPE. Responses to CCh were seen in 90±6% (N=6, n=270) of cells. Unlike the responses described in Chapter 3 for cells in the VZ of the neural retina, the CCh-evoked responses of RPE cells consisted of a large and long-lasting (~200s) rise in base-line \([\text{Ca}^{2+}]_i\), with only small, superimposed oscillatory events (Figure 6.9a). Atropine (10\mu M) and pirenzipine (25\mu M) reduced the number of cells responding to CCh by 80±6% (N=3, n=150, P<0.01, paired Student’s t-test) and 41±10% (N=3, 150, P<0.01, paired Student’s t-test) (Figure 6.9d) respectively, compared to controls, indicating that the response is mediated via mAChRs. No further attempt was made to characterise the sub-type of mAChR involved. Purinergic agonists induced changes in \([\text{Ca}^{2+}]_i\) in a smaller proportion of cells. 36±8% (N=5, n=230) and 21±4% (N=4, n=180) of cells responded to ATP and UTP (100\mu M), respectively (Figure 6.9c). Suramin (25\mu M) reduced the number of cells responding to ATP by 84±1%, compared to that seen in controls (N=3, n=150, P<0.01, paired Student’s t-test) (Figure 6.9e). GABA (100\mu M; N=6, n=270) and glutamate (100\mu M; N=3, n=120) had little effect on \([\text{Ca}^{2+}]_i\) with less than 1±0.5% and 0.5±0.5% of cells, respectively, showing \([\text{Ca}^{2+}]_i\) changes in response to the two agonists (Figure 6.9c).
II. Dye-coupling of neural retinal and pigment epithelial cells in the developing chick eye

The Ca\(^{2+}\) imaging experiments presented in Part I demonstrate the presence of [Ca\(^{2+}\)]\(_i\) waves that occur in both the neural retina and the RPE of the E5 chick retina. These events were occasionally seen to propagate between the two tissues. Waves were rarely seen in the presence of the gap junction blocker carbenoxolone indicating a role for gap junction-mediated transfer of these signals. Investigations in the neocortex indicate that the domains of cells involved in co-ordinated [Ca\(^{2+}\)]\(_i\) activity may be coupled by gap junctions (Yuste et al., 1992, Peinado et al., 1993). The results in this section describe tracer coupling between cells in the neural retina, between cells in the RPE and between these two tissues. In this study, cells in E5 chick retinae were filled with a combination of FITC-dextran (MW 3,000), a dye that cannot pass through gap junctions and remains trapped in the injected cell and Neurobiotin (MW 323), a small tracer molecule that diffuses through most gap junctions and into coupled cells (Mills and Massey, 2000). The results obtained are discussed in the light of the patterns of [Ca\(^{2+}\)]\(_i\) activity seen in the RPE and the neural retina described in Part I.

6.3.6. Cells in the neural retina can be dye-filled using whole cell patch clamping

Attempts to label cells in the neural retina using sharp electrodes had a high failure rate and injected cells showed little in the way of tracer coupling. This was probably due to a combination of damage and difficulty in obtaining stable penetration of cells. Confocal sections through the retina show that numerous processes surround the cell bodies located at the ventricular surface (Figure 6.10c). The use of patch-clamp electrodes permitted cells to be filled through whole cell patching of the fine processes located at the ventricular surface. Cells could be held in whole-cell configuration for several minutes and dye-filled without obvious damage. Dye filling was monitored during patching using a mercury arc-lamp and an FITC filter set. The locations of the filled cells were recorded on a diagram of the retina. A typical FITC-filled cell from an E5 retina is shown in Figure 6.10a. The nucleus is located deep within the retina and processes extend to both the VZ and the GCL. This morphology is identical to that of many cells labelled with Dil using the biolistic technique (see Chapter 5), and is typical
of both retinal PCs and NDCs (Sauer, 1935; Seymour and Berry, 1975; Nagele and Lee, 1979). Histochemical processing shows that, while FITC-dextran is restricted to the injected cells, Neurobiotin is found not only in the injected cell, but also in the cell bodies of many surrounding cells (Figure 6.10d). On occasion, more than one cell in a cluster was labelled with FITC-dextran (Figure 6.10b). These multiple fills probably resulted from the formation of a seal between the patch pipette and more than one VZ process (Figure 6.10c).

6.3.7 Dye-coupling results from diffusion of Neurobiotin through gap junctions

Whilst the most likely explanation for tracer coupling is the movement of Neurobiotin through gap junctions, it is possible that Neurobiotin staining could result from tracer leakage from the electrode and its uptake by neighbouring cells. However, the gap junction blocker carbenoxolone (100μM) prevented the diffusion of Neurobiotin beyond the injected cell (0.4±0.2 cells were labeled with Neurobiotin alone in the presence of carbenoxolone, N=3, n=8; Figure 6.10d). When retinae were injected both before and during perfusion with carbenoxolone, cells filled before application of the drug showed strong coupling, while those injected in its presence did not show coupling (compare Figure 6.10d i and ii). Furthermore, Neurobiotin-stained clusters comprise of a column of stained cells that span the retina; within these columns, dark profiles (i.e. unlabelled cells) are clearly outlined by the stained cells that surround them (see Figure 6.11a). This pattern of staining is unlikely to be explained by a leak of Neurobiotin from the pipette. In experiments where a seal was obtained with the patch electrode, but attempts to rupture the membrane to enter whole-cell mode failed, Neurobiotin staining was absent. Together, these experiments show that Neurobiotin used in the way described only passes into cells coupled to the injected cell by gap junctions.

6.3.8 In the E5 retina clusters of coupled cells largely consist of PCs

Injecting VZ cells with the FITC-dextran and Neurobiotin mixture resulted in the labelling of the injected cell(s) with both FITC-dextran and Neurobiotin, and the cells coupled to it with Neurobiotin (Figure 6.11a). Results described in Chapter 3 show that the early embryonic retinal VZ is composed of dividing PCs, PCs in interphase and
NDCs. In addition, as shown in Figure 6.10c, numerous cell processes terminate at the ventricular surface, and are apparently intermingled with the cell bodies located in the VZ. Mitotic PCs round up and retract their vitreal process (see Chapter 5) while PCs in interphase have a bipolar morphology. NDCs, which can also have a bipolar morphology, can be identified by TuJ-1, an antibody for neuron-specific β-tubulin (Moody et al., 1989; Lee et al., 1990), which has been shown to label ~1/3 of the VZ interphase cell population at E6 (see Chapter 3). In order to further examine the cellular composition of the cluster of coupled cells described above, some dye filled retinae were subsequently processed for β-tubulin using the TuJ-1 antibody.

3D reconstruction of sections through the retina revealed that Neurobiotin stained clusters of cells were columnar in nature, the column spanning the retinal thickness (Figures 6.10d, 6.11a and c). Given the morphology of individual cells, as shown in this thesis and by others, this suggests that many cells within the column may span the thickness of the retina. The injected, FITC-filled cell was usually centrally located with respect to the surrounding column. Occasionally, the Neurobiotin-stained column tapered off towards the GCL. The tapering of the column may reflect the absence of coupled cell somata at the lower levels of the retina, but could also be due to the reduced levels of Neurobiotin at long distances from the injection site.

Individual xy sections through the retina show that, within a cluster boundary, there are many Neurobiotin positive (Neurobiotin⁺) cells both in the VZ and deep in the retina. However, some cells within such a column are Neurobiotin negative (Neurobiotin⁻) and were seen as clearly delineated, dark profiles (Figure 6.11a) with processes that could, by virtue of the stained cells around them, be followed for some distance. These Neurobiotin⁻ cells almost always stained positive² for TuJ-1 (TuJ-1⁺) (Figure 6.11b and c), indicating that they were differentiating or differentiated cells, rather than PCs. Despite the presence of many NDCs at the ventricular surface (see Figure 3.1, Chapter 3), only a small number of TuJ-1⁺ cells were found within Neurobiotin-labelled clusters. Rather, the majority of tracer-coupled clusters appear to consist largely of cells that do not stain for the neuronal marker and are thus likely to be PCs. Surprisingly, few cells

² A Neurobiotin⁻/TuJ-1⁺ cell was classed as one that stained for TuJ-1 and in which Neurobiotin was found at the level of the nucleus.
filled with FITC-dextran stained for TuJ-1, a result that may reflect the rapid withdrawal of VZ processes by NDCs prior to migration, which would reduce the likelihood of these cells being injected. It is conceivable but unlikely that the absence of TuJ-1 staining may result from the dye-filling process causing a disruption of the neuronal tubulin in an injected NDC. Whilst the majority of coupled cells were TuJ-1+, some cells within a Neurobiotin-stained column stained positive for both TuJ-1 and Neurobiotin (Figure 6.11b and c, open arrows). These Neurobiotin+/TuJ-1+ cells were found both near the ventricular surface and deep in the retina. In the neocortex, NDCs are thought to uncouple from PCs prior to migration out of the VZ (Bittman et al., 1997) but form new gap junctions with other immature neurons (Yuste et al., 1992, Peinado et al., 1993). Previous investigations in this laboratory showed that in the retina, RGCs are not only extensively coupled to each other, but sometimes also to other cells of a bipolar appearance, similar to that of PCs and NDCs and which span the depth of the retina (see figure 3 in Becker et al., 2002).

Within the boundary of a cluster, many cells located in the VZ are tracer coupled. Since ~20% of cells in the VZ are in some stage of mitosis at any given time (see Chapter 4) it is possible that some of these cells are coupled to cells within the clusters described above. Patching directly on to mitotic cells proved difficult and most attempts also labelled PCs deeper in the retina with FITC-dextran. This probably resulted from the formation of a seal between the patch pipette and other VZ processes in addition to the targeted mitotic cell. Only 3 dye fills were made in which mitotic cells alone were filled with FITC-dextran and Neurobiotin. In these experiments, no coupled cells were found (Figure 6.12). However, since these experiments were carried out, further work has shown that at least some mitotic cells within the boundary of coupled clusters are Neurobiotin+ (M. Catsicas, personal communication).

6.3.9. RPE cells are dye-coupled

Connexin proteins have been found in the RPE of many species, including chick (Janssen-Bienhold et al., 1998; Becker and Mobbs, 1999) and the Ca2+ imaging experiments described in Part I showed that RPE [Ca2+]i waves are inhibited by gap junction blockers. The techniques described above were used to investigate whether RPE cells are tracer-coupled during the early stages of development. Typical examples
where a single RPE cell was filled with FITC-dextran and Neurobiotin (Figure 6.13a, see also Figures 6.15) show strong Neurobiotin coupling between the injected cell and its neighbours. The number of cells coupled to any injected cell varied but the smallest number observed was 34 and the population of coupled cells often filled the whole field of view (>150 cells using x63 magnification) (n=59). No coupling between RPE cells was observed when injections were made in the presence of carbenoxolone (100µM, n=7, N=3) (Figure 6.13). 3 cells filled before the application of carbenoxolone in the same retinae were extensively coupled to their neighbours (compare Figure 6.13 i and ii). Thus, tracer coupling between cells in the RPE is likely to be mediated via gap junctions.

6.3.10. Connexin staining is found at the interface between the RPE and the VZ

Cx43 is the first Cx protein to be expressed in the eyecup (Becker et al., 1998; Becker and Mobbs, 1999). Antibodies raised to Cx43 proteins have been used to examine the distribution of this gap junction protein within the E5 RPE and the neural retina (Figure 6.14). At E5, Cx43 staining was concentrated at the interface between the RPE and the neural retina at its periphery (Figure 6.14a, arrow heads). At higher magnification, plaque-like labelling between RPE cells (Figure 6.14b, open arrows) and punctate staining between RPE cell processes and the ventricular processes of cells in the neural retina can be seen (Figure 6.14b). Some punctate and plaque-like staining was also found at the interface between mitotic cells and the RPE (Figure 6.14b, asterisk). Within the neural retina, sparse punctate labelling was present within most regions.

6.3.11. Dye coupling occurs between cells of the RPE and the neural retina.

It has been suggested that the RPE can influence cell proliferation in the neural retina (Ilia and Jeffery, 1999; Chapter 4, this thesis). EM studies have shown that gap junctions are located at the interface between the neural retina and the RPE (Dixon and Cronley-Dillon, 1974; Hayes, 1977) although a functional pathway has yet to be

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3 These immunolabelling experiments were carried out by DL Becker and are incorporated here to show that Cx43 gap junction proteins are appropriately located to explain some of the patterns of dye coupling and Ca²⁺ activity described in this thesis.
demonstrated. A series of dye-fills were made in E5 retinas to reveal any coupling between the RPE and the neural retina.

Extensive dye coupling was observed between the RPE and neural retina in the early embryonic eye. Typically, the filled RPE cell was coupled to many surrounding RPE cells and also to a column of Neurobiotin stained cells within the neural retina (Figure 6.15a). The strongest coupling of RPE cells to the retina was usually to cells immediately below the filled cell. These observations are based on the examination of more than 30 cells in 10 retinas. Injections made in the RPE in the presence of carbenoxolone failed to reveal coupling to any neighbouring RPE cells or cells in the neural retina (N=2, n=6; Figure 6.15b) indicating that the tracer coupling between the RPE and the neural retina is mediated via gap junctions. Some retinas were subsequently processed for TuJ-1 staining (data not shown; M. Catsicas, personal communication). Cells in the neural retina that were tracer-coupled to the RPE were usually TuJ-1+. Neurobiotin+/TuJ-1+ profiles were occasionally found although most clusters apparently lacked any Neurobiotin+/TuJ-1+ profiles. Thus, RPE-coupled columns in the neural retina consist predominantly of PCs.
6.4 Discussion

Gap junctional communication has been implicated in many stages in the development of the eye (for review Becker et al., 1998), brain and other tissues (for reviews see Guthrie and Gilula, 1989; Fulton, 1995; Rorig and Sutor, 1996). The experiments described above demonstrate the presence of gap junction-coupled clusters of cells during the first week of development in the embryonic chick neural retina. The majority of cells within these clusters are TuJ-1⁺, indicating that coupled clusters consist largely of PCs. Spontaneous \([\text{Ca}^{2+}]_i\) transients propagate as a wave within local cellular assemblies that include cells within the VZ and throughout the depth of the immature retina. The gap junction blocker carbenoxolone blocks both tracer coupling and the occurrence of wave-like events. Similarly, gap junctions couple RPE cells. Spontaneous \([\text{Ca}^{2+}]_i\) waves also occur within the RPE and are inhibited by carbenoxolone. Cx43, the first connexin to be found in the retina (Becker and Mobbs, 1999), is found at the interface between the RPE and the neural retina. Furthermore, extensive tracer coupling occurs between the RPE and clusters of cells in the neural retina. The gap junctions responsible for this may provide a pathway for \([\text{Ca}^{2+}]_i\) transients or other signals to pass between these two tissues. Preliminary experiments done by others (D.L. Becker, personal communication) suggest that coupling between the neural retina and the RPE declines with age.

6.4.1 Tracer-coupling between cells in the neural retina

Injecting dyes into the fine processes of cells at the ventricular surface of the retina labels cells with a PC-like appearance. These are tracer-coupled by gap junctions to cells that span the thickness of the retina. The resulting columnar organization of coupling between retinal cells described here is similar to that seen between cells in the neocortical VZ (LoTurco and Kriegstein, 1991; Bittman et al., 1997) where dye-coupled clusters span the cortical VZ and have been reported to include between 15-90 cells (LoTurco and Kriegstein, 1991). Such estimates may fall short of the extent of cortical PC coupling, since the tracer used by LoTurco and Kriegstein, Lucifer Yellow (MW 457), only passes through a subset of neuronal gap junctions (Peinado et al., 1993; Mills and Massey, 2000). For he technical reasons discussed below, the number of cells within coupled clusters was not assessed in the experiments described in this chapter.
Neurobiotin is thought to pass through most retinal gap junctions (Mills and Massey, 2000). Following the injection of a cell, the retina was left for 30 mins for the Neurobiotin to diffuse. This period is sufficient to reveal extensive coupling in other situations (LoTurco and Kriegstein, 1991; Penn et al., 1994; Bonness, 1998; Becker et al., 2002). However, the extent of the dye's spread is dependent upon the series resistance of the electrode, which amongst other factors will determine the quantity of Neurobiotin passing into the patched cell. Further, although Neurobiotin staining delineated many individual cell profiles when viewed in xy sections through the region near the site of dye injection, staining of more distant regions was sometimes faint. The apparent size of the clusters of cells may be limited by the diffusion of Neurobiotin and the threshold for detection of the Cy3-Neurobiotin signal with the confocal microscope. Thus, accurate quantification of the number of cells per cluster was not possible. The clusters of retinal cells labelled with Neurobiotin may not be discrete, but rather the coupled cells may form part of a syncytium. If cells were coupled in discrete columnar units, a proportion of the injected FITC-dextran labelled cells would be expected to be located on the periphery of some clusters. Instead, Neurobiotin staining usually surrounded a centrally located FITC-filled cell.

Coupling between cells in the developing retina is not promiscuous. Within the columns of cells revealed by Neurobiotin staining, there were many dark profiles (Neurobiotin-) representing cells that were not coupled to the surrounding cells. These dark profiles were almost always labelled by TuJ-1 directed against the post-mitotic neuronal marker β-tubulin and therefore appear to represent a population of differentiated neurons. While Neurobiotin clusters contained small numbers of few TuJ-1+ cells, the vast majority were TuJ-1-, suggesting that these clusters are comprised largely of PCs. This suggestion requires confirmation through immunolabelling of PCs with a suitable marker such as an antibody directed at vimentin or nestin. In the cortex, NDCs uncouple from cells in the VZ at the final division and remain uncoupled during migration (Bittman et al., 1997), until reaching the cortical plate where they form new and distinct gap junction-mediated connections with other differentiated cells (Peinado et al., 1993; Kandler and Katz, 1995). The uncoupled, TuJ-1+ cells found here in the retina may be NDCs in the process of migration to their final location. The predominant cell type born at the time at which these injections were made (E5) is the RGC (Prada et al., 1991), but
these profiles could also represent other differentiated cell types such as bipolar, horizontal or amacrine cells. Interestingly, previous work in this laboratory in which dye injections were made into immature RGCs has shown that RGCs are tracer coupled to a small population of cells that have a bipolar morphology extending into the depth of the retina (Bonness, 1998; Becker et al., 2002). This population of cells was not stained for TuJ-1 and it is not known whether or not they represent NDCs or PCs. However, if they represent NDCs then the gap junction coupling between them and more mature RGCs could be a precursor to the coupling necessary for the waves of spontaneous \([\text{Ca}^{2+}]_{\text{i}}\) activity seen in RGCs before, and during, synaptogenesis (Catsicas et al., 1998; Wong et al., 1998). In tissue cultures, cell-cell contacts have been shown to be important in the auto-regulation of RGC numbers (Austin et al., 1995; Waid and McLoon, 1998).

Given that TuJ-1\(^+\) cells account for ~25% of all cells (30% of the interphase population) in the retinal VZ at E6, it is perhaps surprising that single, FITC filled cells were always TuJ-1\(^+\). This could be explained if NDCs lose contact with the ventricular surface at or soon after division, prior to moving out of the VZ. The patching procedure might therefore preferentially target PCs whose processes remain at the ventricular surface. Alternatively, it is possible that some of the FITC-filled cells are NDCs but that the patching process somehow damages neuronal \(\beta\)-tubulin, leading to a loss of TuJ-1 staining.

Neurobiotin-stained cells within the clusters were sometimes labelled by TuJ-1. Three hypotheses may account for the presence of Neurobiotin\(^+\)/TuJ-1\(^+\) cells in clusters that consist largely of PCs. Firstly, it is possible that this could result from an artefact of the dye injection process but, given that Neurobiotin coupling was absent in the presence of carbenoxolone, this seems unlikely. Second, it could reflect a small population of NDCs that retain gap junctions with PCs that may be lost at later times.

Coupling between PCs in the neocortex is dynamic and dividing cells uncouple from the rest of the VZ population during mitosis (Bittman et al., 1997). This may also be the case in the retina, since FITC/Neurobiotin-filled mitotic cells showed no spread of Neurobiotin into neighbouring cells. \(\text{Ca}^{2+}\) imaging experiments (see below) also show
that co-ordinated \([\text{Ca}^{2+}]_i\) activity in the VZ usually excluded mitotic cells, even though these cells frequently display individual spontaneous \([\text{Ca}^{2+}]_i\) transients (see Chapter 3). Similar observations have been made in the neocortex (Owens, unpublished observations referenced in Bittman et al., 1997). However, whole-cell patching in the VZ seldom resulted in the labelling of mitotic cells. This was probably due to the forest of small processes that overlie the cell bodies at the ventricular surface (a problem also encountered in the cortex by Bittman et al., 1997). The apparent lack of tracer coupling between mitotic cells and other PCs could also occur if gap junctions were disrupted during the attempt to inject them with dye. Experiments carried out by others (M. Catsicas, personal communication) have since indicated that at least some mitotic cells within the boundaries of coupled clusters are Neurobiotin⁺.

### 6.4.2 Potential roles for coupling between neural retinal PCs

The presence of the extensive gap junctional coupling of neighbouring cells in the neural retina demonstrated above may provide a means by which local groups of PCs can exchange developmentally relevant signals. The observation that only small numbers of NDCs were found in these clusters is strongly suggestive of a developmental role for this process. A specific developmental function for gap junction mediated coupling in the chick retina is supported by the dynamic changes seen in connexin expression (Becker et al., 2002). In the cortex, VZ clusters decrease in size with increasing age (LoTurco and Kriegstein, 1991) indicating that coupling correlates with periods of active proliferation. Thus, coupling through specific connexins may be important in maintaining the proliferative cycle.

Mitotic cells may uncouple from the rest of the PC cluster during division in the cortex (Bittman et al., 1997). Uncoupling during division may be a physical requirement since dividing PCs retract their process and round up at the ventricular surface in order to divide. In the cortex, it has been suggested that cells re-couple during G₁ but that as development progresses more cells remain uncoupled in the later stages of the cell cycle (Bittman et al., 1997). The lack of coupling in G₁ may be necessary for entry into G₀ and differentiation. It would appear that in the cortical VZ coupling between cells positively influences their entry into S-phase. Given that entry into S-phase represents a commitment to divide, coupling therefore appears to act as a positive proliferative
signal. Further experiments will be necessary to show firstly, whether or not mitotic retinal cells remain coupled to other PCs throughout division and secondly, if similar age-dependent patterns of cell cycle stage-specific uncoupling occur.

Many of the cells that form coupled clusters in the cortical VZ are clonally related (Cai et al., 1997). The variation in cell cycle times between cortical PCs of the same age is remarkably small, showing less than 8% variation (Cai et al., 1993, 1995, 1997); clonal descendents progress through the cell cycle in synchrony and form tight clusters (Cai et al., 1997). Clones of daughter cells in the retina also have a columnar structure (Reese and Tan, 1998). Whilst the cortical VZ clusters identified by tracer coupling (LoTurco and Kriegstein, 1991; Peinado et al., 1993) are much larger than the clonal clusters described either in the cortex (Cai et al., 1997) or the retina (Reese and Tan, 1998), coupling between adjacent cells in clonal clusters may facilitate synchronized entry into the different phases of the cell cycle. Signals that pass through the gap junctions between cells within these clusters may act to reinforce contiguity and synchronicity. [Ca^{2+}], could represent one such messenger.

6.4.3 Dye-coupling within the RPE and between the RPE and the neural retina

Gap junctions have been located in the membranes of cultured human RPE cells (Oguni et al., 1991) and EM studies show the presence of gap junctions between RPE cells in the adult eye (Fujisawa et al., 1976), suggesting that in the adult RPE cells are likely to be dye-coupled. Cx43 is the only connexin expressed in the RPE (Becker et al., 1995; Janssen-Bienhold et al., 1998) and immunocytochemical studies presented here show that Cx43 is expressed extensively in the E5 RPE (see also, Becker et al., 1998) and at the interface between the RPE and the neural retina. Injections of FITC-dextran and Neurobiotin showed that not only are RPE cells functionally coupled to one another during early development, but also that extensive tracer coupling occurs between the RPE and the underlying neural retina. Coupling between RPE cells was widespread over the period examined (E4-6). Thus, gap junction communication may provide a mechanism for communication between developing RPE cells.

The demonstration here of tracer coupling and gap junction-mediated communication between the RPE and the developing neural retina is interesting. The potential for such
coupling was indicated by EM studies that describe gap junctions located at the interface of the RPE and the neural retina in Xenopus laevis (Dixon and Cronley-Dillon, 1974; Hayes, 1976) and adult Rhesus monkeys (Townes-Anderson and Raviola, 1981). Previous investigations in this laboratory have indicated that Cx43 may mediate RPE-neural communication in the chick eye (Becker and Mobbs, 1999). The intense Cx43 staining found at the interface between these two tissues further supports this hypothesis. At E5, RPE cells were frequently coupled to cells in the underlying neural retina. The tracer-coupled clusters in the neural retina were very similar in appearance to those observed following injections made into the isolated neural retina and also consisted of predominantly, TuJ-1 progenitor-like cells (M. Catsicas, personal communication). Thus, RPE-neural retinal coupling appears to be strong at a time when proliferative divisions are high (Prada et al., 1991).

6.4.4 Potential roles for coupling between the RPE and the neural retina

PCs divide in the VZ, adjacent to the RPE, and thus the RPE is in an ideal position to influence PC division. Studies of the albino retina have demonstrated a strong regulatory role for the RPE in controlling proliferation in the neural retina (Ilia and Jeffery, 1996; for review, see Jeffery, 1997). The lack of pigmentation in albino eyes is due to defects in the melanin synthetic pathway, usually in the enzyme tyrosinase. The maturation of the neural retina in albinos is delayed and a period of increased cell proliferation is followed by increased levels of apoptosis. Dopa, a precursor in the melanin synthetic pathway, may be an important factor in the normal regulation of proliferation (Ilia and Jeffery, 1996) and can cause RPE cells to arrest in G1/S-phase (Akeo et al., 1992). Thus, some factor associated with melanin synthesis appears to act as a brake on retinal proliferation. Precisely how this effect is mediated is unclear. Dopamine, a product of dopa via the action of dopa decarboxylase, has been shown to regulate gap junctions via a cAMP-dependent pathway in the mature retina (Vaney, 1994; Mill and Massey, 1995). It is possible that dopamine could regulate gap junction coupling between neural retinal PCs and the RPE. The low levels of dopa found in albino retinae may alter communication between the RPE and the neural retina, allowing higher levels of gap junctional communication beyond the time when they would normally become more restricted in pigmented animals. The imaging studies carried out here show that other neurotransmitter systems act on the developing RPE to
cause changes in $[Ca^{2+}]_i$. A variety of neurotransmitters, including ACh and GABA, are known to modulate gap junctions through 2nd messenger pathways (for review, see Rorig and Sutor, 1996). Further work is required to establish what role, if any, dopa or other neurotransmitter systems have in the regulation of gap junctional coupling between the RPE and the neural retina.

The analysis of mutant mice has identified several genes that control the lamination of the cortex during development (for review, see Rice and Curran, 1999). The processes involved in the regulation of retinal lamination appear to be fundamentally different to those in the cortex. This may be because of the unique association of the developing retina with the RPE, and other tissues such as the lens. It has been proposed that the physical presence of RPE cells is required to establish the polarity and laminar structure of the retina (Vollmer et al., 1986; Layer et al., 1998). RPE cells induce an ordered laminar organisation within the spheroids that form in retinal cultures (Vollmer et al., 1986; Layer et al., 1998). Selective ablation of the RPE in intact eyes causes loss of retinal lamination in the lesioned regions (Raymond and Jackson, 1995). It is conceivable that gap junction-mediated communication between the RPE and the neural retina is important in the lamination process. However, whilst knock down of Cx43 in the chick has been shown to cause a reduction in the level of proliferation and the production of small eyes, the laminar organisation of the neural retina is not affected (Becker and Mobbs, 1999).

6.4.5 Co-ordinated $[Ca^{2+}]_i$ activity in the RPE and neural retina

Whilst a variety of developmental roles have been shown or suggested for gap junction communication, few experiments have addressed the nature of the signals that are exchanged via them. $Ca^{2+}$ and other low molecular weight 2nd messengers, such as IP$_3$, can all diffuse through gap junctions (Saez et al., 1989). The imaging studies presented here show that $[Ca^{2+}]_i$ signals can spread within neural retinal and RPE cells and, albeit rarely, between the two tissues. The wave-like events in both the RPE and the neural retina were markedly reduced by the gap junction blocker carbenoxolone. Another gap junction blocker, halothane has been shown to block mechanically stimulated $[Ca^{2+}]_i$ waves in rat RPE cell cultures (Stalmans and Himpens, 1997). Similar $[Ca^{2+}]_i$ events
have been observed in the neocortical VZ (LoTurco and Kriegstein, 1999; Owens and Kriegstein, 1998).

The [Ca^{2+}] waves in the neural retina described above propagated at a rate of ~15μm/s (range 7-41μm/s), which is similar to that seen between retinal glial cells (~23μm/s; Newman and Zahs, 1997), and in the range of speeds demonstrated for gap junction-mediated Ca^{2+} signal transfer found in the cortex (Yuste et al., 1995; Owens and Kriegstein, 1998). The mechanism by which carbenoxolone blocks gap junctions is unknown, and it is possible that, like octanol and halothane, the agent may have effects on other membrane proteins (e.g. Murrell et al., 1991; Jones et al., 1992). Nonetheless, the results described above, together with the fact that the waves occur at a time when extensive tracer coupling is seen and carbenoxolone blocks the spread of Neurobiotin, strongly suggests that gap junctional communication is involved in mediating coordinated activity in the neural retina and the RPE. In the RPE, tracer coupling extended over much larger regions than the [Ca^{2+}] waves. The signals responsible for initiating, propagating and limiting the size of waves is unknown. The [Ca^{2+}] waves observed in the neural retina and RPE are initiated by one or several trigger cells, and propagated out at a velocity of ~15μm/s. This is considerably slower than the regenerative [Ca^{2+}] events seen in the GCL during synaptogenesis (150-300μm/s), and is more likely to represent simple diffusion of Ca^{2+}, or possibly IP_{3}, from a central source since the diffusion constant of buffered Ca^{2+} inside cells is ~13μm/s (Allbritton et al., 1992, for review, see Rüttingen and Iversen, 2000).

Extensive gap junction-mediated coupling between the RPE and the neural retina is shown in Part II of the results section of this chapter. On a couple of occasions [Ca^{2+}] transients were seen to propagate between the two tissues. Spontaneous [Ca^{2+}] activity was observed propagating between RPE cells and then into the neural retina, and on one occasion a wave originating in the neural retina appeared to propagate into the RPE. The speed of propagation and the extent of gap junction-mediated coupling between these tissues are consistent with the possibility that these events propagate via the spread of Ca^{2+} or some other Ca^{2+}-releasing 2nd messenger through gap junctions between the RPE and the neural retina. However, the very low frequency at which these events were observed is surprising given the extensive tracer coupling between the RPE.
and the neural retina. However, \( \text{Ca}^{2+} \) may not be important in the communication between these two tissues and a 2\(^{nd}\) messenger such as cAMP may instead be involved. Establishing the precise mechanisms and nature of the transmitted signals between these tissues will require further investigation.

6.4.6 The RPE response to neurotransmitters

Whilst there has been extensive investigation of the role of neurotransmitters in the developing retina, few studies have examined their effects on the developing RPE. Imaging experiments in this chapter demonstrate that the isolated E5 chick RPE responds to both purinergic and muscarinic stimulation with a change in \([\text{Ca}^{2+}]_i\). A full pharmacological characterisation of the receptor subtypes involved in these responses was not attempted. However, both ATP and UTP caused marked increases in a large proportion of RPE cells, and this response was blocked by suramin. Thus, these results indicate that P2Y receptors are involved. P2Y and P2X receptors are thought to mediate the biphasic response to ATP seen in cultured rat (Ryan et al., 1999; Stalmans and Himpens, 1997), bovine (Peterson et al., 1997) and human (Mitchell, 2001) RPE cells. ATP is understood to bind to P2Y receptors causing an IP\(_3\)-mediated release of \( \text{Ca}^{2+} \) (Stalmans and Himpens, 1997) and an initial release of ATP, which then feeds back on to P2X receptors causing an augmented release of ATP (Mitchell, 2001). Since suramin is a rather non-specific antagonist it is possible that both P2Y and P2X receptors are present on chick RPE cells. The pharmacological characterisation of purinergic receptors is far from straightforward. The use of specific P2X and P2Y receptor antagonists will be required to define the precise nature of the purinergic receptors involved.

Application of CCh also caused large increases in \([\text{Ca}^{2+}]_i\) in the isolated E5 RPE. These responses were blocked by atropine and reduced by pirenzipine suggesting that mAChRs are involved. Given the only partial inhibition by pirenzipine, more than one subtype of mAChR may be present. A partial characterisation of the muscarinic response in human RPE cells suggests that the response is mediated via receptors of the M\(_3\) subtype (Feldman et al., 1991). Immunocytochemical studies in newly hatched chicks showed staining in the RPE for cm2, cm3 and cm4 receptors.
Immunocytochemical studies and further pharmacological investigation will be required to establish the developmental profile of mAChR subtype expression.

The function of the mAChRs and P2-receptors expressed in the RPE during development is unknown. That mAChR-binding sites are present in high density in the RPE during the developmental period but decline thereafter (Salceda, 1994) supports the notion that these receptors are of developmental importance. It is possible that neurotransmitters and their receptors are involved in the initiation of the spontaneous $[\text{Ca}^{2+}]_i$ waves seen in the RPE (see above). Such activity may represent a mechanism for external factors to co-ordinate activity both between groups of cells within the RPE and between the RPE and neural retina. $\text{Ca}^{2+}$ activity could pass directly into adjacent cells through gap junctions. Alternatively, or perhaps in addition to gap junction-mediated transfer, the rise in $[\text{Ca}^{2+}]_i$ may be sufficient to cause the release of ATP or other regulatory factors. ATP has been shown to increase $[\text{Ca}^{2+}]_i$ and induce auto-release of further ATP from human RPE cells (Mitchell, 2001). The results presented in Chapter 4 show that the RPE appears to influence proliferation in the neural retina by a mechanism involving purinergic receptors. The results described in this thesis, and by others, have shown that both purinergic stimulation and $[\text{Ca}^{2+}]_i$ signals are important in cell cycle regulation.
6.5 Further studies

The results described in this chapter raise interesting questions about the function of gap junctional coupling both between retinal PCs, and between the RPE and the neural retina during development. It will be important to establish the role of the RPE in regulating neural retinal proliferation, and any part that gap junctions may play in this mechanism. A novel technique for driving connexin expression has recently been developed (Becker et al., 2001). This approach, involving the introduction of constructs that either up- or down-regulate connexin expression, could be used to investigate the effects of changing the extent of gap junctional communication between the RPE and the neural retina on retinal PC proliferation and differentiation.

The role of co-ordinated spontaneous [Ca\textsuperscript{2+}]i activity in the RPE and its importance in the regulation of the development of the neural retina requires further investigation. Given the effects of the albino mutation on the development of the retina, it would be interesting to probe the effects of this mutation on Ca\textsuperscript{2+} signalling, ATP release and the cell cycle using imaging techniques such as those developed for the investigations described above. Specifically, given the suggestion that dopa may be an important factor in the regulation of proliferation in the retina, it would be interesting to investigate the effects of this agent on Ca\textsuperscript{2+} signalling and gap junctional coupling.
6.6 Summary

Studies of albino animals (Ilia and Jeffery, 2000) suggest that the RPE may play an important role in the regulation of neural retinal proliferation. The experiments presented in this chapter were designed in order to better understand the physiological relationship between the neural retina and the RPE. The pattern of gap junctional coupling and its role in Ca²⁺ signalling in these two tissues was examined. The results show that at E5 RPE cells are coupled via gap junctions both to one another and to PCs in the neural retina. Cells within the neural retina form gap-junction coupled clusters that consist largely of PCs and essentially exclude NDCs. This segregation of coupling between PCs and NDCs may provide a mechanism by which the RPE can directly influence actively proliferating cells in the underlying neural retina. Changes in [Ca²⁺]ᵢ in the RPE and neural retina were monitored. These experiments show that periodic spontaneous Ca²⁺ signals propagate via gap junctions in both the RPE and neural retina and that, albeit rarely, Ca²⁺ signals pass between the two tissues. Ca²⁺ transients propagated as waves that invaded many cells in the RPE while in the neural retina Ca²⁺ activity was more spatially restricted. Results in chapter 4 showed that the presence of the RPE and purinergic agonists and antagonists had profound effects on the rate of mitosis in the neural retina that may be mediated by ATP release from the RPE and purinergic receptors present on dividing cells in the neural retina. It is conceivable that co-ordinated Ca²⁺ activity in the RPE is required for the release of ATP into the subretinal space. Alternatively, gap-junctional coupling between the RPE and neural retina may provide a direct pathway for other signalling molecules to pass between these two tissues and allow the RPE to influence proliferative cell divisions via a Ca²⁺-independent mechanism.
Figure 6.1. Spontaneous $[\text{Ca}^{2+}]_i$ waves in the E5 RPE. A, Single confocal section through the RPE of a Fluo-4 loaded retina. Regions of coordinated activity occurring over a 10min period have been outlined (red or black). Arrows indicate the cell initiating a given wave. The $[\text{Ca}^{2+}]_i$ activity in the ROIs circled in (lower right hand corner) are shown in B. Changes in $\Delta F/F$ in the cells labelled in (A) show the propagation of the wave out from cell 2. Cells 5 and 6 undergo an apparently unrelated paired change just before the onset of the wave. C, A time-lapse series of single confocal images of a region within an E5 RPE loaded with Fluo-4 showing a spontaneous $[\text{Ca}^{2+}]_i$ wave. Arrow indicates the cell initiating the wave. Scale bars 20µm.
Figure 6.2. Spontaneous [Ca$^{2+}$]$_i$ transients in the E5 RPE. A, example traces of spontaneous changes in [Ca$^{2+}$]$_i$ (ΔF/F) in individual RPE cells, at 36°C. B, Frequency distribution of the number of transients occurring in cells that demonstrated spontaneous activity during a 500s imaging period. Graph shows the mean±SEM, N=3, n=117.
Figure 6.3. Mitotic cells are rarely involved in spontaneous $[Ca^{2+}]$, waves in the VZ. Interphase and mitotic cells were identified from Hoechst images (Ci) taken before and after a Ca$^{2+}$ imaging series. Cii shows a region from a single image within the imaging period. Traces of $\Delta F/F$ (A and B) show that a coordinated event occurs at 220s and involves many interphase cells but only one mitotic cell, which is in prophase (cell 1). Other coordinated events (highlighted by dashed lines on A and B) occur during the imaged period but did not incorporate dividing cells. Spontaneous single $[Ca^{2+}]$ transients are indicated (red arrows). Scale bars on traces: single line 25%, and double line 50%, $\Delta F/F$ respectively. Scale bar on C 10µm.
Figure 6.4. Spontaneous $[\text{Ca}^{2+}]_i$ waves in the E5 VZ. A single xy scan of the VZ of an E5 retina loaded with Fluo-4 in the absence of RPE, taken from a movie. The image shows regions of co-ordinated spontaneous $[\text{Ca}^{2+}]_i$ activity (outlined) occurring within a 500s imaging period. The cell from which each wave was initiated is indicated by an arrow coloured the same as that used to indicate the wave’s boundary. Two regions, outlined in bold, were active at the time this single image was taken (the region outlined in bold yellow is shown in detail in Figure 6.3). Within the boundaries of active regions, mitotic cells were often excluded from the coordinated rise in $[\text{Ca}^{2+}]_i$. One example is highlighted at the bottom where the red line traces the boundary of a $[\text{Ca}^{2+}]_i$ wave and open arrows indicate mitotic cells both within and at the edge of the boundary, which did not participate in the wave (Hoechst image not shown). Scale bar $10\mu m$. 
Figure 6.5. Co-ordinated spontaneous 
$[\text{Ca}^{2+}]_j$ activity involves cells throughout the depth of the neural retina. A, time series of a confocal ‘virtual z-section’ through the depth of the neural retina in an E5 retina labelled with Fluo-4. Images are pseudo-coloured; warm colours indicate higher $[\text{Ca}^{2+}]_j$. B, Example traces of $\Delta F/F$ from the 6 cells indicated in (A). 18 cells were involved in this event and 4 cells within the boundaries of this active group did not show a coordinated change in $[\text{Ca}^{2+}]_j$. Scale bar 10μm.
Figure 6.6. Spontaneous $[\text{Ca}^{2+}]_i$ activity in the E5 VZ is higher when the RPE is in place. A, histogram showing the percentage of cells undergoing spontaneous changes in $[\text{Ca}^{2+}]_i$ in the presence or absence of the overlying RPE in a 500s period. B, histogram showing the number of transients individual cells underwent in the presence or absence of the RPE, in a 500s period. Note that the number of events/500s in any given cell increases in the presence of the RPE as well as the proportion of cells demonstrating spontaneous activity. N=3, n=150; **P<0.01
**Figure 6.7.** Propagation of a spontaneous \([\text{Ca}^{2+}]_i\) wave from the neural retina to the RPE. Such events were rare. A, single confocal section showing a ‘virtual z-section’ through the VZ and RPE of an E4 retina which was loaded with Fluo-4 and imaged at 36°C. B, A combined Hoechst 33342 (blue) and Fluo-4 (green) image of the same area as in A. The background levels of the Fluo-4 signal have been reduced in this image so that the Hoechst signal is clearly visible. Line indicates the level of the VZ. C, traces of \(\Delta F/F\) within the ROIs indicated by the circles, showing the propagation of activity from the neural retina to the RPE. Scale bar=10µm
Figure 6.8. \([\text{Ca}^{2+}]_i\) activity propagates from the RPE to the VZ. A transient moves from a cell in the RPE before traversing the length of a single neuroepithelial cell. A, ‘virtual z-scans’ of a Fluo-4 loaded E5 retina. The active neuroepithelial cell (dashed lines) and three ROIs have been highlighted in (i). B, Traces of the ROIs indicated in (Ai). Scale bar=10\(\mu\)m.
Figure 6.9. The E5 RPE responds to purinergic and muscarinic stimulation. A, example traces of changes in ΔF/F in individual RPE cells during application of CCh (100μM). B, example traces of changes in ΔF/F in individual RPE cells during application of ATP (100μM). C, histogram showing the proportion of RPE cells responding to ATP (N=5), UTP (N=4), CCh (N=6), GABA (N=6) and glutamate (N=3) (all 100μM). Results are mean percentage ± SEM. D, histogram showing the effects of atropine (grey stripes; 10μM; N=3, n=150) and pirenzipine (light grey; 25μM; N=3, n=150) on the response to CCh (100μM). E, histogram showing the effects of suramin (25μM; N=3, n=150) on the response to ATP (100μM). Results in (D) and (E) are mean percentage ± SEM, compared with the control response.
Figure 6.10. Dye coupling in the neural retina. A, a typical neuroepithelial profile, filled with FITC-dextran using a patch-pipette. B, multiple FITC fills resulting from patching onto the terminal processes that terminate at the outer edge of the VZ. C, a cross-section of a region in an E5 retina stained with Bodipy-ceramide (red), which labels cell membranes, and Hoechst (green). Open arrows show ventricular processes of cells located below the VZ, which surround a mitotic cell. Scale bar 5μm. D, Carbenoxolone prevents movement of Neurobiotin into coupled cells. i, dye injection in control solution (FITC dextran-yellow) shows large numbers of coupled cells (Neurobiotin-red) and ii, dye injection in the presence of carbenoxolone leads to staining of fewer cells and little coupling (Arrow indicates one weakly labelled Neurobiotin+ cell). Injected cells appear yellow because they contain both FITC (green) and Neurobiotin (red). Scale bars 10μm, except in C.
Figure 6.11. Dye coupling of cells in the E5 neural retina. Ai and ii, single xy confocal sections at the level of the two dashed lines in (Aiii) which shows the Neurobiotin (red) staining surrounding dye-injected cell (yellow). Note the dark holes indicating that some cells remain uncoupled despite being surrounded by Neurobiotin (examples highlighted with arrows) ii, xz projection, through cells labelled following injection of FITC-dextran and Neurobiotin into neuroepithelial cells. B and C, Many NDCs appear to be excluded from coupled clusters. B, single xy confocal section through a coupled cluster, taken mid-way through the thickness of the retina i, Neurobiotin staining (red) surrounds dark, uncoupled cells; ii, TuJ-1 staining (blue) of the same cells shown in i; iii, overlay image (red and blue channels) of Bi and ii shows that uncoupled cells are usually TuJ-1⁺ (small arrows, also indicated in Bii). Occasionally, TuJ-1⁺/Neurobiotin⁺ cells were observed (open arrows). C, overlay images of single xy confocal sections through a column of coupled cells showing the pattern of TuJ-1 and Neurobiotin staining at different levels. The dye-injected cell is labelled yellow and a TuJ-1⁺/Neurobiotin⁺ stained cell is indicated by an open arrow. Scale bars 10μm.
Figure 6.12. Mitotic cells may not be coupled to other PCs A, FITC-filled mitotic cells did not show Neurobiotin coupling with adjacent cells i, FITC-filled cell (yellow) ii, cross-section of Hoechst 33342 labelling at level of dashed line in i, shows the injected cell to be mitotic iii, combined image of the mitotic chromatin (blue) and surrounding Neurobiotin (red). Scale bars 5 μm. Figure reproduced with permission from P. Bayley.
Figure 6.13. RPE cells are coupled by gap-junctions. Carbenoxolone prevents movement of Neurobiotin into coupled cells Ai, dye injection in control solution (FITC dextran-yellow) shows large numbers of coupled cells (Neurobiotin-red) and ii, dye injection made in the same retina in the presence of carbenoxolone shows no coupling to adjacent cells. Scale bar=10μm.
Figure 6.14. Cx43 staining (green) in E5 retina which has been double labelled with propidium iodide (red). A, an xy projection of 5 confocal sections (taken at 1μm intervals). Cx43 staining is strong at the boundary between neural retina and RPE (e.g. arrow heads). B, high power image (x63 magnification with x2 digital zoom) shows Cx43 plaques occur between neighbouring RPE cell processes (open arrows) as well as between the neural retina and the RPE (arrow heads). Occasional Cx43 plaques between mitotic profiles in the VZ and RPE cells (asterisk). Images provided by D.L. Becker.
Figure 6.15. RPE cells are coupled with the neural retina. Ai, FITC-filled RPE cell in E5 retina, showing coupling with its neighbouring pigment cells ii, xz projection of area shown in (Ai), taken through the depth of the RPE and neural retina. Dye-injections (FITC dextran-yellow) made in cells in the RPE shows large numbers of coupled cells (Neurobiotin-red) and B, dye injections made in the same retina in the presence of carbenoxolone prevented the spread of Neurobiotin into both adjacent RPE cells (Bi) and the neural retina (Bii). Scale bars 10 μm.
Chapter 7

Conclusions

During CNS development it is essential that appropriate numbers of neurons and glia are produced to establish normal brain function. It is well established that the regulation of both proliferation and differentiation depends on the interactions of diverse extracellular signal molecules linked to a network of intracellular transduction pathways. There is growing evidence for the involvement of a variety of both slow and fast neurotransmitters in the regulation of cell proliferation. Further, $[\text{Ca}^{2+}]$, has a key influence on developmental events in the CNS and has been implicated in the regulation of differentiation, migration, cell fate and circuit formation.

This thesis demonstrates that before synapses are formed, cells in the VZ of the chick retina display intermittent spontaneous $[\text{Ca}^{2+}]$, transients. These transients are independent of action potential production, as they are in the cortex (Owens and Kriegstein, 1998; Owens et al., 2000). However, in contrast to the cortex, $\text{Ca}^{2+}$ activity in the retinal VZ depends on transmitter release. Comparison of the changes in $[\text{Ca}^{2+}]$, with the pattern of chromatin in cells in the VZ shows that GABAAergic and glutamatergic receptors are present within a primarily interphase population of cells. The combined use of Ca$^{2+}$ imaging, staining of chromatin with Hoechst 33342, and immunocytochemistry for neuronal $\beta$-tubulin (TuJ-1), demonstrates that GABA receptors are predominantly expressed by a population of differentiating neurons. In contrast, muscarinic receptors are almost ubiquitous at E6, suggesting that these receptors play important roles in both differentiating and progenitor cells at this time. The rise in $[\text{Ca}^{2+}]$, in response to GABA and glutamate is blocked by Ni$^{2+}$ and thus likely results from a Ca$^{2+}$ influx via VGCCs, whereas that in response to muscarinic and purinergic stimulation is suppressed by caffeine, frequently oscillatory in nature, and therefore likely to result from the release of Ca$^{2+}$ from intracellular stores rather than Ca$^{2+}$ influx.

The expression of transmitter receptors by PCs changes with time; whereas most mitotic and interphase cells respond only to CCh, and not UTP, at E6, most cells
respond to both agonists at E4. The functional significance of this change in the pattern of receptor expression with time is unknown, but it is possible that it correlates with a switch from symmetrical division of PCs at early times in development, necessary for a rapid increase in the number of cells in the progenitor pool, to increased numbers of asymmetric divisions, necessary for creating large numbers of differentiated cells at later times (Desai and McConnell, 2000).

Direct imaging of part of the mitotic process shows that both muscarinic and purinergic stimulation may significantly affect the rate of mitosis in the retina. Muscarinic stimulation acts as a brake on mitosis at metaphase, almost doubling the time it takes for chromosomes to separate, while purinergic stimulation acts as an accelerator, reducing the time taken for this process to a third. These effects of muscarinic and purinergic stimulation do not appear to be compensated for later in the cell cycle since the presence of UTP, carbachol and pirenzipine for a longer period have marked effects on eye size consistent with the short-term effects of these agents on mitosis. It is conceivable that the transmitter-evoked release of Ca\(^{2+}\) from stores within PCs exerts a direct effect on the rate of mitosis. \([\text{Ca}^{2+}]_i\) signals have been shown to be important in controlling cell cycle elsewhere (for review see Whitaker and Larman, 2001). Whilst GABA and glutamate are also able to produce increases in \([\text{Ca}^{2+}]_i\), the Ca\(^{2+}\) influx through VGCCs that they evoke does not appear to regulate mitosis.

Throughout the proliferative cell cycle PCs migrate back and forth between the VZ and the vitreal side of the retina by the process of INM. The “DiOlistic” technique was used to label cells in the VZ and examine the characteristics of INM. PC nuclear movement is saltatory, characterised by alternations between movements in a forward or backward direction interspersed with stationary periods. Preliminary investigations indicate that \([\text{Ca}^{2+}]_i\) transients occur in cells during INM, which may be important in its regulation. Surprisingly, neurotransmitters had little effect on the speed of PC INM.

In the cortex, clusters of ventricular cells are coupled via gap junctions (LoTurco and Kriegstein, 1991) although the function of this coupling is poorly understood. Here, in the chick retina, gap junctions couple clusters of neural retinal cells that consist
predominantly of PCs rather than NDCs. Extensive dye coupling also occurs between cells in the RPE and the neural retina. The function of this pathway is unclear but it may be important in the regulation of proliferation. The chick RPE displays frequent, spontaneous and coordinated changes in $[\text{Ca}^{2+}]_i$ transients and expresses both purinergic and muscarinic receptors. On occasion this activity was seen to spread to the underlying neural retina. The RPE had a profound influence on the rate of cell proliferation in the neural retina, an effect that may be mediated through the release of ATP and other factors.
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290


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