Purinergic signalling during zebrafish development and the characterisation of novel fish mutants

William Howard James Norton

Submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

May 2002

Department of Anatomy and Developmental Biology
University College London
University of London
Acknowledgements

Many thanks to Steve, Nigel and Geoff for their supervision, patience and help over the last three and a half years.

I am indebted to Klaus, Lukas and Claire for all the technical and spiritual advice that they gave me in the wilderness years! Thanks also to Masa and Zsolt for helpful discussions of experiments and results.

Lab life would have been much more dull without constant distractions from Filipa, Diz, Marika, Florencia, Rich, Astrid, Jacqui, Elise and Dave. Thanks also to Paul, Lucy and Arantza for thesis writers' solidarity over the last few months and to Tom for endless games of Backgammon. I hereby admit that he is a better Backgammon player than me!

Thanks to all the people in Tübingen who looked after the London screen team and showed us the sights around town.

Special thanks go to Sónia, Adam, Alastair and Becky for being great friends and listening to constant complaints for the last few years. I would not have finished this thesis without their help.

Finally, thanks to my Mum and Dad, who have always supported and believed in me. This thesis is dedicated to them.
ABSTRACT

Purinergic signalling during zebrafish development and the characterisation of novel fish mutants

This thesis looks at two different aspects of zebrafish development and as such is divided into two main sections.

The first section looks at the expression of novel purinoceptors during zebrafish development. Purinoceptors are receptors that are activated by either adenosine or ATP and its analogues. They have been shown to have important roles during the development of many species. The thesis looks at the expression of three new zebrafish homologues, two of which are ionotropic receptors ($p2x3$ and $p2x4$) and one of which is a G protein coupled receptor ($p2y11$), both in wild-type embryos and embryos of selected mutants. The results suggest roles for these receptors during development and show that the fish is a good model to study early developmental expression patterns. Experiments to elucidate the function of one of the genes were performed.

The second part of the thesis reports the finding and characterisation of novel zebrafish mutants. A large scale ENU screening strategy was used to identify a large number of fish with axonal pathfinding defects at 36 hours of development, using an anti-acetylated tubulin antibody. The embryos were screened for defects in the axon scaffold formation. The final results chapter describes the characterisation of monorail, a zebrafish midline mutant that shows floorplate defects. A combination of midline marker gene expression pattern analysis and mapping of the mutation to known single sequence length polymorphisms (SSLPs) allows the mutated gene to be identified.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>1</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>2</td>
</tr>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Table of contents</td>
<td>4</td>
</tr>
<tr>
<td>List of figures</td>
<td>15</td>
</tr>
<tr>
<td>List of tables</td>
<td>17</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>18</td>
</tr>
<tr>
<td><strong>CHAPTER 1. General Introduction</strong></td>
<td>22</td>
</tr>
<tr>
<td>1.1. The zebrafish</td>
<td>22</td>
</tr>
<tr>
<td>1.1.1. The fish as a developmental model</td>
<td>22</td>
</tr>
<tr>
<td>1.2. Mutagenesis screens</td>
<td>23</td>
</tr>
<tr>
<td>1.3. Receptors for extracellular nucleotides</td>
<td>26</td>
</tr>
<tr>
<td>1.3.1. History of purinergic signalling</td>
<td>27</td>
</tr>
<tr>
<td>1.3.2. The P1 receptor family</td>
<td>29</td>
</tr>
<tr>
<td>1.3.3. The P2 receptor family</td>
<td>30</td>
</tr>
<tr>
<td>1.3.4. P2X receptors</td>
<td>31</td>
</tr>
<tr>
<td>1.3.5. P2X receptors as heterotetramers</td>
<td>33</td>
</tr>
<tr>
<td>1.3.6. P2X$_1$ receptor</td>
<td>34</td>
</tr>
<tr>
<td>1.3.7. P2X$_2$ receptor</td>
<td>34</td>
</tr>
<tr>
<td>1.3.8. P2X$_3$ receptor</td>
<td>35</td>
</tr>
<tr>
<td>1.3.8.1. Tissue distribution of the P2X$_3$ receptor</td>
<td>36</td>
</tr>
<tr>
<td>1.3.8.2. P2X$_3$ receptors are involved in the response to pain</td>
<td>37</td>
</tr>
<tr>
<td>1.3.8.3. P2X$_3$ receptors on sensory nerve terminals are heterotetramers</td>
<td>38</td>
</tr>
<tr>
<td>1.3.8.4. P2X$_3$ receptor expression in the enteric nervous system</td>
<td>39</td>
</tr>
<tr>
<td>1.3.8.5. P2X$_3$ receptors expressed in myelinated pelvic afferents</td>
<td>39</td>
</tr>
</tbody>
</table>
1.3.8.6. Hyporeflexia in P2X₃ knockout mice 40
1.3.8.7. Models of inflammatory disease 40
1.3.8.8. Pharmacology of the zebrafish P2X₃ receptor 40

1.3.9. P2X₄ receptor 42
1.3.9.1. Agonists 42
1.3.9.2. Antagonists 43
1.3.9.3. Modulation by metal ions 45
1.3.9.4. Tissue distribution of the P2X₄ receptor 45
1.3.9.5. Central nervous system 45
1.3.9.6. Other tissues 46
1.3.9.7. Thyroid 46
1.3.9.8. Bladder 46
1.3.9.9. Heart tissue expressed P2X₄ 47
1.3.9.10. Xenopus oocytes 48
1.3.9.12. Vascular tissues 48
1.3.9.12. Retina 49
1.3.9.13. Expression in other tissues 49

1.3.10. P2X₅ receptor 49
1.3.11. P2X₆ receptor 50
1.3.12. P2X₇ receptor 50
1.3.13. P2Y receptors 51
1.3.14. P2Y receptor orphans 54
1.3.15. P2Y₁ receptor 54
1.3.16. P2Y₂ receptor 55
1.3.17. P2Y₄ receptor 56
1.3.18. P2Y₆ receptor 56
1.3.19. P2Y₁₁ receptor 57
   1.3.19.1. Pharmacology 57
   1.3.19.2. Activation of two signalling pathways 58
   1.3.19.3. Molecular characteristics 59
1.3.19.4. Stimulation of renin in human kidney cells 60
1.3.19.5. Tissue distribution 61
1.3.19.6. P2Y₁₁ function in HL-60 cells 61
1.3.19.7. P2Y₁₁ receptors stimulate secretion in pancreatic cells 62
1.3.20. P2Y₁₂ receptor 63
1.3.21. P2Y₁₃ receptor 63

1.4. Release and removal of ATP during purinergic signalling 64
   1.4.1. Release of ATP into extracellular space 64
   1.4.2. Breakdown and removal of ATP 66

1.5. P2 receptors during development 67
   1.5.1. P2 receptors can regulate cell growth and differentiation 68
   1.5.2. P2 receptors can regulate apoptosis during development 69
   1.5.3. Early roles for nucleotides in embryogenesis 70
   1.5.4. Chick P2Y₁ 70
   1.5.5. Frog P2Y₈ 71
   1.5.6. Limb bud development 72
   1.5.7. Retina development 72
   1.5.8. Otocyst development 72
   1.5.9. Branchial arch and somite development 73
   1.5.10. Skeletal muscle development 73
   1.5.11. Mesonephros development 74
   1.5.12. P2 receptors in postnatal development 74
   1.5.13. Brain expression during development 75
   1.5.14. Heart and vasculature development 75
   1.5.15. Lung development 76
   1.5.16. Gastrointestinal tract development 76
   1.5.17. Vas Deferens development 77
   1.5.18. Development in other tissues 77

1.6. Summary 78

CHAPTER 2. Materials and Methods 80
2.1. Fish maintenance and care 80
   2.1.1. Observation of live embryos 80
   2.1.2. Generation of transgenic fish and double mutant lines 81

2.2. Molecular biology techniques 81
   2.2.1. Novel P2 receptor genes 82
   2.2.2. Bacterial stocks 82
   2.2.3. Primer design 82
   2.2.4. Polymerase chain reaction 83
   2.2.5. Reverse transcription polymerase chain reaction 83
   2.2.6. Sequencing 83
   2.2.7. Tree diagrams 84

2.3. Embryological techniques: Detection of gene and protein expression 84
   2.3.1. Preparation of DNA templates 84
   2.3.2. Transcription of RNA probes 84
   2.3.3. Wholemount in situ hybridisation 85
   2.3.4. Wholemount double in situ hybridisation 86
   2.3.5. Plastic sections of wholemount in situ hybridisations 87
   2.3.6. in situ hybridisations on cryostat sections 87
   2.3.7. cDNAs used for in situ hybridisations 88

2.4. Immunohistochemistry on wholemount embryos 88
   2.4.1. Antibody staining 88
   2.4.2. Backfills of hindbrain and reticulospinal neurons 90

2.5. ENU mutagenesis protocols 90
   2.5.1. Generation of founder fish - ENU mutagenesis 90
   2.5.2. Mutant crossing scheme 91
   2.5.3. Screening protocol, UCL screen 91
   2.5.4. Screening protocol, Tübingen screen 2000 93
   2.5.5. Analysis of fluorescent antibody and GFP expression 94

2.6. Preparation of morpholinos and RNA for injection 94
   2.6.1. Cloning of foxtail into PCS2+ 94
2.6.2. Ligation of prepared templates into PCS^{2+}  
2.6.3. *In vitro* transcription of mRNA for injection  
2.6.4. Preparation of morpholinos for injection  
2.6.5. Injection of morpholinos and RNA  
2.7. Mapping of genes  
2.8. Photography and image analysis  

**CHAPTER 3. Embryonic expression of a P2X_{3} receptor in zebrafish**  
3.1. Abstract  
3.2. Introduction  
3.3. Pharmacology  
3.4. Tissue distribution of the P2X_{3} receptor  
3.5. Results  
   3.5.1. Molecular characteristics  
   3.5.2. *In situ* hybridisation expression pattern  
   3.5.3. Double labelling of P2X_{3} positive cells  
   3.5.4. Functional analysis  
   3.5.5. Morpholino injection and design  
   3.5.6. Limitations of morpholinos  
   3.5.7. Morpholino against P2X_{3}  
   3.5.8. Phenotype of morphant embryos  
   3.5.9. Tubulin staining on morphant embryos  
   3.5.10. Patch clamping of fish trigeminal ganglia  
   3.5.11. Touch responsiveness of injected vs uninjected embryos  
   3.5.12. Responsiveness of embryos incubated in P2X_{3} blockers  
   3.5.13. Single application of agonist causes an escape response  
   3.5.14. Application of other agonists caused no escape response  
   3.5.15. Agonist application to embryos incubated in antagonists  
3.6. Discussion  
   3.6.1. Expression of P2X_{3} in fish  
   3.6.2. A suggested common origin of P2X_{3} expressing cells  
   3.6.3. Species differences between expression pattern of P2X_{3}
7.3.2. Other factors in the Nodal signalling pathway 220
7.3.3. Nodal signalling in ventral midline formation 222
7.3.4. cyclops and squint 222
7.3.5. one-eyed-pinhead 223
7.3.6. schmalspur 224

7.4. The Hedgehog signalling pathway 224
7.4.1. Introduction 224
7.4.2. Short-range signalling in the Hedgehog pathway 225
7.4.3. Long-range signalling in the Hedgehog pathway 226
7.4.4. Zebrafish Hedgehog pathway mutants provide insights into the activity of Hedgehog in patterning the central nervous system 229
7.4.5. smoothened 229
7.4.6. sonic-you 230
7.4.7. detour 230
7.4.8. you-too 231
7.4.9. Expression of the Hedgehog ligands in midline tissue during development 232
7.4.9.1. sonic hedgehog 232
7.4.9.2. tiggywinkle hedgehog 233
7.4.9.3. echidna hedgehog 233
7.4.9.4 Table of hedgehog gene expression 233
7.4.10. Redundancy of Hedgehog signalling in midline patterning 234
7.4.11. Summary - a two step model of floorplate induction 234

7.5. Other zebrafish floorplate mutants 236
7.5.1. iguana 236
7.5.2. schmalhans 238
7.5.3. monorail 238

7.6. Function of the floorplate during development 238
7.6.1. Long-distance guidance cues 239
7.6.2. Netrin 240
7.6.3. Bone morphogenetic protein 7 241
7.6.4. Short-range guidance cues 241
7.6.5. axonin-1, NrCAM and NgCAM 242
7.6.6. F-spondin 242
7.6.7. Altered responsiveness of growth cones 244
7.6.8. Semaphorins 245
7.6.9. Ephrins and Eph receptors 246
7.6.10. roundabout and slit 247

7.7. Mapping of novel mutants using SSLPs 249

7.8. Results 250
7.8.1. Phenotype of monorail 250
7.8.2. Mapping of the gene encoding monorail 250
7.8.3. Width of the floorplate in the midbrain and hindbrain 252
7.8.4. Expression of foxa1 in mol embryos 253
7.8.5. Medial floorplate in induced but not maintained in mol 253
7.8.6. Lateral floorplate is not formed in mol embryos 258
7.8.7. mol embryos show a reduction of gene expression in the basal plate of the midbrain as well as in the midline floorplate 258
7.8.8. Loss of the floorplate in mol causes patterning defects 261
7.8.9. Other markers show defects in mol embryos 266
7.8.10. mol embryos have a reduction of specific neurons in the midbrain and isthmic region of the brain 269
7.8.11. Endodermal genes are normally expressed in mol 272
7.8.12. foxa1 acts downstream of two signalling pathways 272
7.8.13. Expression of shh and twhh in smoothened mutants 277

7.9. Discussion 277
7.9.1. Phenotypic summary 277
7.9.2. Region specific maintenance of the floorplate during development 282
7.9.3. Migration and specification of neuronal number is intact in monorail 282
7.9.4. Tubulin staining shows subtle defects in mol embryos 283
7.9.5. Induction of 5HT and CNIII nuclei in the midbrain 284
7.9.6. *hedgehog* family genes induce branchiomotor neurons at discrete location along the anterior-posterior axis 285
7.9.7. *fox* gene function in other species 286
7.9.8. Partial redundancy of *foxa2* function in *mol* embryos may underlie the ventral neural tube patterning defects 286

7.10. Conclusions 287
    7.10.1. Future experiments 287

CHAPTER 8. General Discussion 289
8.1. Part 1: The role of P2 receptors during zebrafish development 289
    8.1.1. P2X<sub>3</sub> may be involved in mediating a sensory response to touch stimulation 289
    8.1.2. Function of the P2X<sub>3</sub> receptor during development 290
    8.1.3. P2X<sub>4</sub> is expressed in the retina, gut and brain during zebrafish development 291
    8.1.4. P2Y<sub>11</sub> expression during development 292
    8.1.5. The zebrafish as a model to study P2 receptor expression during development 293
    8.1.6. Future plans 293

8.2. Part 2: Screening and characterisation of novel zebrafish mutants 294
    8.2.1. *monorail* suggests novel roles for *forkhead box* genes during development 295
    8.2.2. Zebrafish *forkhead box* genes have different roles in midline patterning 295
    8.2.3. *foxa1* and *foxa2* have similar expression patterns and regulatory pathways 296
    8.2.4. Future plans 296

CHAPTER 9. Bibliography 298
Appendix 1 345
Appendix 2 346
List of Figures

1.1. Summary of zebrafish developmental stages 25
1.2. Diagram of a P2X receptor 32
1.3. Diagram of a P2Y receptor 53
2.1. ENU mutagenesis breeding scheme 92
3.1. Tree diagram showing P2X receptor homologies 101
3.2. Early expression of p2x3 in zebrafish 104
3.3. Late expression of p2x3 in zebrafish 106
3.4. Morpholino design 110
3.5. p2x3 morpholino injected embryo phenotype 112
3.6. Tubulin staining on morphant embryos 114
3.7. WT embryos stimulated with a von Frey hair 116
3.8. Morphant embryos stimulated with a von Frey hair 117
3.9. PPADS incubated embryos embryos stimulated with a von Frey hair 118
3.10. Water application to WT embryos 120
3.11. ATP solution application to WT embryos 121
3.12. ADP solution application to WT embryos 122
3.13. Adenosine solution application to WT embryos 123
3.14. pH4 solution application to WT embryos 124
3.15. ATP solution application to TNP-ATP blocked embryos 125
4.1. Sequence alignment of p2x4 paralogues 140
4.2. Tree diagram comparing p2x4 homologies 143
4.3. p2x4 expression in zebrafish 144
4.4. p2x4 staining on cryo-section of zebrafish larvae 147
4.5. p2x4 staining on bonnie and clyde mutants 150
4.6. p2x4 staining on faust mutants 151
4.7. p2x4 staining on casanova mutants 153
5.1. Tree diagram comparing P2Y receptor homologies 161
5.2. Sequence alignment of \( p2y11 \) paralogues

5.3. Early expression of zebrafish \( p2y11 \)

5.4. Late expression of zebrafish \( p2y11 \)

6.1. Tubulin and opsin antibody staining on WT embryos

6.2. \textit{wingnut}

6.3. \textit{akineto}

6.4. Table showing Tübingen screen 2000 mutants

6.5. Class 1 brain mutants

6.6. Class 2 brain mutants

6.7. Class 3 brain mutants

6.8. Class 4 brain mutants

7.1. Cartoon depicting Nodal pathway signalling

7.2. Cartoon depicting Hedgehog pathway signalling

7.3. Hedgehog genes induce branchiomotor neurons in the hindbrain

7.4. Hedgehog patterns the ventral neural tube

7.5. Floorplate patterns the ventral neural tube

7.6. Phenotype of \textit{monorail}

7.7. \textit{foxa1} expression in \textit{monorail}

7.8. Medial floorplate is induced but not maintained in \textit{monorail}

7.9. Lateral floorplate is not induced in \textit{monorail}

7.10. Expression of midline genes is reduced in brain of \textit{monorail}

7.11. \textit{monorail} shows bilateral patterning defects

7.12. Other midline defects in \textit{monorail}

7.13. \textit{monorail} lacks specific neurons in the midbrain and hindbrain

7.14. Endodermal genes are expressed normally in \textit{monorail}

7.15. Expression of \textit{foxa1} in \textit{mzoep} and \textit{smoothened}

7.16. Expression of \textit{shh} and \textit{twhh} in \textit{smoothened}

7.17. Diagram of \textit{foxa1} activity in midline signalling
List of Tables

Table 1. Description of mutants found in Tübingen screen (Fig 6.4.) 198
Table 2. Expression of *hedgehog* genes in the zebrafish midline 233
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP binding-cassette protein</td>
</tr>
<tr>
<td>AB-MECA</td>
<td>N(\text{6}-(4\text{-amino-3-}\text{[125}\text{i}]\text{iodobenzyl})\text{adenosine-5-N-methylcarboxamide}))</td>
</tr>
<tr>
<td>AC</td>
<td>Anterior commissure</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAEC</td>
<td>Bovine alveolar epithelial cell</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2 bis (0-aminophenoxy)ethane-N,N,N',N'tetraacetic acid</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
</tr>
<tr>
<td>CCPA</td>
<td>2 chloro-N(^6)-cyclopentyl-adenosine</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane receptor</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovarion cell</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPX</td>
<td>p-Xylene-bis(N-pyrimidinebromide)</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DVDT</td>
<td>Dorso-ventral diencephalic tract</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EGF-CFC</td>
<td>Epidermal growth factor - colony forming cells</td>
</tr>
<tr>
<td>ENU</td>
<td>Ethynitrosourea</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethylmethane sulphonate</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-amino butyric acid</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial derived neurotrophic factor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney cell</td>
</tr>
<tr>
<td>HL-60</td>
<td>Human Leukocyte-60 cell</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatic nuclear factor</td>
</tr>
<tr>
<td>HPF</td>
<td>Hours post-fertilisation</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human vascular endothelial cell</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IDI</td>
<td>Idiopathic detrusor instability</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5 triphosphate</td>
</tr>
<tr>
<td>ITP</td>
<td>Inosine 5'-triphosphate</td>
</tr>
<tr>
<td>LFP</td>
<td>Lateral floorplate</td>
</tr>
<tr>
<td>LLF</td>
<td>Lateral line fasciculus</td>
</tr>
<tr>
<td>LLG</td>
<td>Lateral line ganglion</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Microtubule associated protein kinase</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madine Darby canine kidney cell</td>
</tr>
<tr>
<td>2meSATP</td>
<td>2-methyl-thio-adenosine triphosphate</td>
</tr>
<tr>
<td>MFP</td>
<td>Medial floorplate</td>
</tr>
<tr>
<td>MHB</td>
<td>Midbrain-Hindbrain boundary</td>
</tr>
<tr>
<td>MLF</td>
<td>Medial longitudinal fasciculus</td>
</tr>
<tr>
<td>MPC</td>
<td>Midline precursor cell</td>
</tr>
<tr>
<td>NaAC</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>NANC</td>
<td>Non-adrenergic, Non cholinergic</td>
</tr>
</tbody>
</table>
NgCAM  
Neural-glial cell adhesion molecule

NGF  
Nerve growth factor

NH₄AC  
Ammonium acetate

NrCAM  
Neuronal cell adhesion molecule

PENECA  
2-(2-phenyl)ethyladenosine-5'-N-ethy luronomamide

PC  
Posterior commissure

PCR  
Polymerase chain reaction

PDEC  
Pancreatic duct endothelial cell

PFA  
Paraformaldehyde

PIP2  
Phosphatidyl inositol (4,5) bisphosphate

PKC  
Protein kinase C

PLC  
Phospholipase C

PND  
Postnatal day

POC  
Posterior commissure

PPADS  
Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate

PTU  
Phenythioarobamide

RNA  
Ribonucleic acid

rtPCR  
Reverse transcription PCR

SOT  
Supraoptic tract

SSLP  
Simple sequence length polymorphism

TAE  
Tris-Acetate-EDTA

TCA  
Trichloroacetic acid

TE  
Tris-EDTA

TGF  
Transforming growth factor beta

TNP-ATP  
2',3'-0-(2,4,6-trinitrocyclohexadienylidine)

TPH  
Tryptophan hydroxylase

TPOC  
Tract of the posterior commissure

VR1  
Vanilloid receptor 1

UDP  
Uridine diphosphate
UTP  Uridine 5'-triphosphate
UV  Ultraviolet light
WIK  Wild India Kalkutta
WT  Wild-type
ZLI  Zona limitans intrathalamica
CHAPTER ONE

GENERAL INTRODUCTION

1.1. The zebrafish

Zebrafish are small freshwater teleosts that originate from the Ganges basin in India. They are a member of the Cyprinidae family. The generic name is *Danio rerio*, which was designated at the 1993 zebrafish meeting at Cold Spring Harbour Laboratory. Older literature uses the original generic name, *Brachydanio rerio*. The first use of the zebrafish as a genetic model was by George Streisinger in Oregon (see Streisinger et al., 1981). He recognised the potential for keeping large numbers of small freshwater fish that have a short breeding time and can produce large clutches of embryos. Developmental stages of the zebrafish have been described by several different labs (Roosen-Runge, 1938, Hisoaka and Battle, 1958, Kimmel, 1989), although it was not until 1981 that Streisinger introduced the fish as a vertebrate model suitable for systematic mutagenesis.

1.1.2. The fish as a developmental model

In contrast to other classical model organisms, the zebrafish allows the simultaneous application of both experimental embryology and genetic analysis to answer specific biological questions. Classical model systems tend to favour only one of these approaches: the frog and chicken are useful for extensive embryological work, whereas the mouse and fruitfly are very powerful genetic model organisms. The zebrafish provides a unique opportunity to study vertebrate developmental biology.
1. General Introduction

Adult zebrafish are relatively small (around 3-4cm long) and reach sexual maturity within three or four months of being born. The fish can be mated every day and produce clutches of up to 100 or 200 embryos that provide easy access to all developmental stages. The size of the fish allows thousands of mutant lines to be stored in a small area. As the fish are inexpensive to maintain and can be bred in large numbers it is possible to carry out genetic screens for mutations that effect embryogenesis (Eisen, 1996).

Zebrafish embryos are large and are fertilised externally. The embryo is clear, allowing both the immediate detection of developmental abnormalities and dye tracing studies to look at the fates of particular cells during development. The advent of green fluorescent protein (GFP) lines in fish has also highlighted the usefulness of transparent embryos. Coupling of a GFP gene to the promoter region of a gene of interest allows the visualisation of the expression of the gene as development progresses. At 24 hours post fertilisation (hpf), all the organ systems present in the adult fish have developed. The beating heart and movement of blood in arteries and veins can be seen. The embryo is responsive to touch and the brain shows clear divisions and specialisations (Fig. 1.1.).

1.2. Mutagenesis screens

The zebrafish is estimated to contain between 800 and 2,800 mutable genes, although only a fraction of these are thought to be required for the survival, shape and patterning of adult, juvenile or embryonic organisms (Solnica-Krezel et al., 1994). Although every gene in an organism is likely to have an important function, however subtle, one of the main goals of developmental biology is to elucidate the genes that have indispensible and unique functions in the early pattern morphogenesis of a fish. One of the best methods that has been employed to do this has been mutant screening, in which single genes are chemically mutated and the resulting phenotype is analysed. Thus, a classical genetic approach can be used to work back and find a gene
1. General Introduction

that has a key role in the correct development of an organ or tissue. Adult male zebrafish are mutated randomly using ethyl nitrosourea (ENU) to cause point mutations in genes. Only a fraction of genes will code for proteins that show

*Figure 1.1*

Camera lucida drawings showing several stages of zebrafish development. The embryo develops on top of a large yolk cell and undergoes a series of meroblastic cleavages to form a ball of cells. During gastrulation, the ball of cells spreads down to cover the yolk. An embryonic organiser, the shield, is formed. This is followed by convergence-extension and then somitogenesis. By the end of the first day the embryo has formed all the organ systems seen in an adult fish and is responsive to touch.

Figure 1.1.

1-cell 0.2 h
16-cell 1.5 h
high 3.3 h
50%-epiboly 5.3 h

bud 10 h
6-somite 12 h
14-somite 16 h

26-somite 22 h
long pec 48 h
1. General Introduction

visible phenotypes and which can then be screened for and recovered. However powerful, genetic screens are subject to several limitations. For instance, redundancy of genes can occur when the removal of one orthologue is masked by the upregulation of function of the other. Other limitations include differences in genes that are seen to be developmentally important in the laboratory and genes that are important in the natural environment. There have been several extremely successful screens for diploid fish mutants. At least three such screens have been carried out: two in Tübingen, Germany and one in Boston, USA (for review see Granato and Nüsslein-Volhard, 1996). The history and design of these genetic screens will be discussed in further detail in chapter 6. Characterisation of a zebrafish midline mutant, monorail, found in the first Tübingen screen will be described in Chapter 7.

Additional information about the genes that underlie developmental processes can be gained by characterising the molecular nature of the gene by cloning. The methods used to map genes rely on the generation of a zebrafish genetic linkage map (Postlethwait et al., 1994). This can be achieved by candidate gene testing or positional cloning. In the candidate gene testing method, the map position of known cloned genes are compared to the map position of the gene of interest. The search can be limited to genes that are expressed in the tissue which is phenotypically altered in the mutant. The positional cloning method identifies a DNA sequence located near the mutation and then sequentially isolates overlapping DNA sequences until the gene of interest is reached (Postlethwait and Talbot, 1997). The function of the genes can be further characterised by injections of sense or anti-sense RNA or the injection of a morpholino against the gene (Ekker, 2000).

1.3. Receptors for extracellular nucleotides

Intracellular nucleotides are known to be important in energy metabolism, nucleic acid synthesis and enzyme regulation. However, after some early
resistance, it is now recognised that some purine nucleotides and nucleosides can also act as extracellular signalling molecules. The aim of the first part of this thesis is to look at the possibility that these signalling molecules may have a role during zebrafish embryonic development. In this chapter, the choice of the zebrafish as a developmental model will be discussed, as well as the history and classification, molecular structure and function of these receptors for extracellular nucleotides.

1.3.1. History of purinergic signalling

The idea that purines may function as signalling molecules in the heart and blood vessels was first proposed by Drury and Szent-Györgyi in 1929. Application of both adenosine and adenosine 5'-monophosphate (AMP) to the mammalian heart were shown to have biological effects (Drury and Szent-Györgyi, 1929) including heart block, arterial dilatation and lowering of blood pressure.

The first hint that ATP might act as a neurotransmitter came from Holton (1959), who showed that the rabbit ear artery releases ATP upon antidromic stimulation of the sensory nerves supplying it. Subsequent work was carried out to show the effects of purine nucleotides on a wide range of functions. Work carried out in the 1960's proposed the existence of a group of non-adrenergic-non-cholinergic (NANC) autonomic nerves that supplied the gastrointestinal tract (Burnstock et al., 1963, Martinson and Muren, 1963). In another study, adenosine was suggested to be the physiological regulator of coronary blood flow during hyperaemia (Berne, 1963). As the evidence for this NANC transmission increased, the race was on to identify other molecules that could act as transmitters in the autonomic nervous system. The compound that emerged as the most likely candidate for this was adenosine 5' triphosphate (ATP, Burnstock, 1972). Application of ATP to smooth muscle of the gastrointestinal tract and bladder mimicked NANC responses, a clear sign of purinergic transmission. ATP satisfied the criteria that had been set out at the
time, to determine whether a substance was acting as a true transmitter (see Eccles, 1964). In 1976 the concept of co-transmission was also introduced (Burnstock 1976a), although it took over ten years for both ideas to be generally accepted. Intense pharmacological analysis followed these observations and it was eventually proposed that both adenosine and its analogue, ATP, could act on receptors in the central nervous system. Receptors have been described as having the ability to "recognise a distinct chemical entity and translate information from the entity into a form that the cell can read to alter its state" (Kenakin et al., 1992). Thus, the actions of adenosine and ATP on both the nervous system and non-neuronal cells had to be as a result of activation of a new class of receptor. Extracellular purines and pyrimidines have have now been shown to act on a wide range of biological processes including smooth muscle contraction, exocrine and endocrine secretion, inflammation, platelet aggregation as well as many others. Specific receptors have been cloned and characterised (see Ralevic and Burnstock, 1998). The signalling has also been shown to play roles during embryological development (Bogdanov et al., 1997, Meyer et al., 1999, Burnstock, 2001b).

This new class of extracellular receptors for purines were named "purinergic receptors" in 1978 and has since be divided into P$_1$-purinoceptors that have adenosine as their main ligand and P$_2$-purinoceptors that use ATP as the main ligand (Burnstock, 1976b). Further research showed that two subtypes of P2 receptor existed. The distinction between the two subtypes was originally proposed after initial pharmacological data showed discrepancies between the affinity of various ATP analogues at the ligand binding site (Burnstock and Kennedy, 1985). P2X receptors were seen to be activated both by ATP and a closely related analogue $\alpha,\beta$ methylene ATP ($\alpha,\beta$ me-ATP). Activation of P2X receptors was seen to mediate vasoconstriction and contraction of smooth muscle. In contrast, P2Y receptors appeared to be potently activated by 2-methylthioATP, and were seen to mediate vasodilatation and relaxation of smooth muscle. There are now known exceptions to this distinction, although
1. General Introduction

subsequent evidence has shown that the two different subtypes do indeed exist. Analysis of molecular data has shown that P2X receptors are ionotropic and P2Y receptors metabotropic.

1.3.2. The P1 receptor family

The first evidence that adenosine may act as an agonist in the body came from the observation, in 1929, that adenosine could have biological effects on the mammalian heart (Drury and Szent-Györgyi, 1929). The actions of caffeine, a P1 receptor antagonist, on the heart has also prompted further research on receptors for adenosine and related ligands.

The P1 family of adenosine activated receptors is currently known to have four cloned members, named $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. Selective agonist and antagonists have now been found for nearly all the subtypes. The endogenous agonist adenosine is quickly metabolised by a large number of enzymes (reviewed in Deussen, 2000), making it a poor tool for the study of adenosine receptors. In order to circumvent this, chemical modification has been used to make stabler, more selective agonists. CCPA is an $A_1$ receptor agonist, PENECA and AB-MECA are agonists at the $A_3$ receptor. P1 receptors are antagonised by a wide variety of compounds including caffeine, naturally occurring xanthine derivates and chemically modified variations of both. MRS 1334 (a dihydropyridine) is selective for the human $A_3$ receptor. SCH58261 is selective for $A_{2A}$ receptors. The therapeutic potential of developing selective agonists and antagonists is extremely large. P1 receptors have been implicated in many physiological situations (Klotz, 2000).

P1 receptors are coupled to G proteins and can modulate adenylyl cyclase activity to change the activity of cyclic AMP (cAMP). The $A_1$ and $A_3$ subtypes of receptors decrease cAMP activity, whilst both $A_{2A}$ and $A_{2B}$ increase cAMP activity. Further research has shown that other cellular responses are also possible, including stimulation of $K^+$ ion conductance (Trussell and Jackson,
1. General Introduction

1985), inhibition of Ca^{2+} ion conductance (Scholz and Miller, 1991), and stimulation of phospholipase C. P1 receptors are seven pass transmembrane domain receptors with extracellular carboxyl and intracellular amino terminals. Adenosine acts as a vasodilator of the heart and as a neuromodulator that suppresses general central nervous system (CNS) functions. Examples of this include the stimulatory effects of both caffeine and theophylline and the inhibition of hippocampal Glutamate (Glu) release.

As the main part of this thesis is based on the study of P2 ATP receptors, the P1 family of receptors will not be discussed in more detail here. Subclassifications and physiological roles of P1 receptors are reviewed by Fredholm et al., 2000, Klotz, 2000 and Ralevic and Burnstock, 1998.

1.3.3. The P2 receptor family

The first overview of the function of ATP as an agonist in various tissues in the body was published by Burnstock and Kennedy (1985). Some P2Y receptor subtypes have since been seen to be able to respond to pyrimidines as well as purines. P2Y_2 and P2Y_4 receptors are potently activated by UTP, and the P2Y_6 receptor is activated by UDP (for review see Ralevic and Burnstock, 1998). Following the initial subdivision into P2X and P2Y receptor subtypes (Burnstock and Kennedy 1985), studies of the molecular structure and the transduction mechanisms used by each subtype has shown that the P2X receptors have two transmembrane domains (Valera et al., 1994 and Brake et al., 1994). P2Y receptors have a typical G protein coupled receptor structure with seven transmembrane domains (Webb et al., 1993, Lustig et al., 1993). Seven mammalian members of the P2X family and six mammalian members of the P2Y family are currently recognised, although there may still be more that have not been cloned in mammals.

Studying purinoceptors has been more difficult than first expected, partly because of a lack of specific agonists and antagonists for most of the different
1. General Introduction

subtypes. P2X receptors have the ability to form heteromultimers in tissues and splice variants of some P2X receptors have been found in several species. The presence of ecto-enzymes that can rapidly degrade ATP to its analogues in solution, has further complicated the study of these receptors (see Khakh et al., 2000, North and Surprenant, 2000). Each subtype of P2 receptor will now be discussed separately.

1.3.4. P2X receptors

There are now seven mammalian members of the P2X family (P2X₁- P2X₇). The subtypes show high homology to each other, ranging from 35% to 48% overall and increasing to around 90% in the transmembrane domains. A P2X₅ subtype has been cloned from chick skeletal muscle (Bo et al., 2000), although no mammalian homologue has yet been found. It is possible that it may turn out to be an orthologue of the P2X₅ receptor. The P2X subunits range in size from 379 to 595 amino acids long.

P2X receptors are ATP activated ligand gated ion channels that have two transmembrane domains. The joining extracellular loop is extensively N-glycosylated and has a characteristic group of ten conserved cysteine residues, fourteen conserved glycine residues and between two and six N-linked glycosylation sites. Both the carboxyl and amino terminals are intracellular and short. The exceptions to this rule are the P2X₂ and P2X₇ receptors, both of which have an elongated C-terminal. The structure of the P2X receptor most closely matches that of an amiloride sensitive epithelial Na⁺ channel. As such, P2X receptors form a novel class of ligand gated ion channels (Fig. 1.2.). The major ligand at the P2X receptor is ATP, with variable responses to other stable ATP analogues such as α,β Me-ATP and β,γ Me-ATP. The binding of the agonist allows the channel to open and allows rapid permeability to cations including Na⁺, K⁺ and Ca²⁺ (Bean, 1992, Dubyak and el-Moatassim, 1993, North
Figure 1.2. Diagram of a P2X receptor. Activation by a ligand allows the channel to open and let ions pass through. See text for full explanation.
1. General Introduction

1996). P2X receptors mediate fast transmission, acting within a few milliseconds. The ligand binds directly to the channel and causes it to open rapidly. An example of this type of transmission is in response to ATP released at nerve synapses. Each of the subtypes of P2X receptors differs in its pharmacology (particularly in the response to $\alpha_1\beta$ Me-ATP and $\beta_1\gamma$ Me-ATP) and tissue distribution. Each subtype will be discussed in detail in the next section.

1.3.5. P2X receptors as heteromultimers

P2X receptors are multisubunit receptors and may act as heteromultimers in tissues. The usual arrangement appears to be a receptor made of three subunits, with homomultimers and heteromultimers having different pharmacological properties. To date, there have been four heteromultimers characterised in expression systems (Surprenant et al., 2000, King et al., 2000). A lot of the information about heteromultimers comes from estimates following SDS-polyacrylamide gel electrophoresis (Nicke et al., 1998). A good example of this is the P2X$_3$ receptors that can act alone (ie as a homomultimer) or in combination with P2X$_2$ subunits (P2X$_{2/3}$ receptor) as a heteromultimer (Torres et al., 1999). Responses recorded from adult sensory neurons following ATP stimulation match those recorded when P2X$_2$ and P2X$_3$ receptors are coexpressed together (Lewis et al., 1995). These heteromultimers may explain the discrepancies seen between recordings from P2X receptors in native and recombinant situations. The P2X$_{2/3}$ channel is not the only P2 receptor heteromultimer to have been found and studied. Evidence also exists for P2X$_{1/5}$ (Torres et al., 1998), P2X$_{2/6}$ (King et al., 2000) and P2X$_{4/6}$ (Lê et al., 1999, see introduction to Chapter 4). These heteromultimers appear to be important for generating functional diversity in ATP-mediated responses.

Alternate splicing of P2X genes and species differences also increases the number of differences seen between receptors. Another factor that can account for changes in the measured responses from P2X receptors is the action of cations that can modulate P2X receptor activity. The sparcity of
1. General Introduction

selective agonists and antagonists has made the characterisation and the elucidation of physiological roles for P2X receptors very difficult. The next section will briefly describe the expression and agonist profiles of each type of P2X receptor. The P2X$_3$ and P2X$_4$ receptors will be discussed in greater detail as novel zebrafish homologues of these genes are described in chapters 3 and 4.

1.3.6. P2X$_1$ receptor

The P2X$_1$ receptor is the most significantly expressed P2X receptor in smooth muscle, although the expression is not uniformly distributed and is developmentally regulated. It is expressed in the smooth muscle layers of the arteries and arterioles, vas deferens, male reproductive tract (Vial and Evans, 2001), at low levels in the spleen and lung as well as in the dorsal root ganglia, the smooth muscle cells of blood vessels and in the urinary tract (Valera et al., 1994). The P2X$_1$ receptor is sensitive to $\alpha,\beta$-meATP and 2MeSATP as well as ATP and is desensitised extremely rapidly-in the space of a few hundreds of milliseconds. Comparison of the pharmacological profile of the receptor in smooth muscle to that recorded from P2X$_1$ channels in expression systems, suggests that it is acting as a homomultimer. ATP released from nerves in tissues such as the vas deferens acts on P2X$_1$ receptors to cause a rapid phasic relaxation of the tissue. Activity on an $\alpha_1$ adrenoceptor in the same tissue will cause a tonic relaxation instead. Mice in which the P2X$_1$ receptor has been deleted show infertility as the response of the vas deferens sympathetic stimulation is much reduced (Mulryan et al., 2000).

1.3.7. P2X$_2$ receptor

The P2X$_2$ receptor was originally cloned from rat pheochromocytoma (PC12) cells (meATP (Brake et al., 1994). It is activated by ATP, 2MeSATP, adenosine and 5'-O-3-thiotriphosphate (ATP$_{\gamma}$S) but is not affected by either $\alpha,\beta$-meATP or $\beta,\gamma$-meATP (Brake et al., 1994). The receptor shows little or no signs of desensitisation. P2X$_2$ receptors are widely expressed in a variety of tissues
including brain, spinal cord and bladder as well as the pituitary gland and vas deferens (Lynch et al., 1999). P2X$_2$ receptors show significant permeability to Ca$^{2+}$ ions and at high Ca$^{2+}$ concentrations the responses of the receptor will be attenuated (Migita et al., 2001). The P2X$_2$ receptor is the only P2 subtype that has been shown to be sensitive to extracellular acidification. A reduction of the extracellular pH to <7.0 causes an increase in the response seen to ATP as an agonist. Zinc (Zn$^{2+}$) has also shown to be a potent potentiator of responses to agonists (King et al., 1996, Wildman et al., 1997, Wildman et al., 1998).

1.3.8. P2X$_3$ receptor

The zebrafish homologue of the P2X$_3$ receptor was characterised as part of this thesis. The tissue distribution and function of the zebrafish homologue will be described in chapter 3. As a result of this, a more detailed report of the pharmacology and tissue distribution of the P2X$_3$ receptor in other species will be given here.

The pharmacological properties of P2X$_3$ receptors in mammals are well documented (see Rae et al., 1998, Wildman et al., 1999, North and Surprenant., 2000). The rat P2X$_3$ receptor, for example, shows higher potency to $\alpha,\beta$ meATP the fish receptor and is inhibited by low concentrations of TNP-ATP (Egan et al., 2000). Allosteric modulators of the rat receptor have also been found. These can enhance the response to binding of the ligand. An example of this is cibacron blue (Jarvis et al., 2001). As this thesis does not aim to study the pharmacological properties of P2 receptors, properties of receptors in other species will not be discussed in great detail here. The changes in the responses to agonists and antagonists can be attributed to changes in the underlying amino acid sequences of the receptor proteins. Several studies documenting the cloning and analysis of paralogues from a wide variety of species allows evolutionary aspects of purinoceptor pharmacology and physiology to be discussed (Seguela et al., 1996, Bogdanov et al., 1998).
1. General Introduction

1.3.8.1 Tissue distribution of the P2X3 receptor

Studies in a wide variety of species, using both antibodies and in situ hybridisations have shown that the P2X3 receptor is expressed in both the enteric and central nervous systems as well as in sensory ganglia. A large number of studies have reported that the receptor has a role in nociceptive function, probably after activation by ATP released from damaged or inflammed tissues.

In the central nervous system of rats, mice and humans, P2X3 expression has been reported in the cell bodies of the dorsal horn on the spinal cord and the nucleus of the tractus solitarius in the medulla oblongata (Llewellyn-Smith and Burnstock, 1998, Yiangou et al., 2000). Expression in the spinal cord is usually restricted to lamina II (Bradbury et al., 1998, Ramer et al., 2001). P2X3 expression has been seen to be induced by application of glial derived neurotrophic factor (GDNF) or nerve growth factor (NGF) to spinal cord preparations. Expression of the receptor was found in lamina I and outer lamina II following GDNF or NGF treatment (Ramer et al., 2001).

Cell bodies of sensory neurons, including the trigeminal, nodose and dorsal root ganglions show P2X3 expression in a subset of small diameter sensory neurons (Chen et al., 1995, Lewis et al., 1995). Electron microscopic analysis shows that the P2X3 receptor expression is concentrated in membrane-bound organelles of the cell including the endoplasmic reticulum and the golgi body (Llewellyn-Smith and Burnstock, 1998). The trigeminal ganglion in many species has two different cell types, an early neural crest derived component and a later formed placodal component (for information about the mouse see Stainier and Gilbert, 1991). P2X3 expression in the trigeminal ganglion of the rat is restricted to small diameter crest-derived sensory neurons (Chen et al., 1995). P2X3 receptor expression has also been reported in taste buds and cochlear and vestibular ganglia of rats (Bo et al., 1999, Xiang et al., 1999). The expression in sensory ganglia co-localises with both the vaniloid receptor 1
1. General Introduction

(VR1, activated by capsaicin), calcitonin gene related peptide (CGRP) and Isolectin IB4 (Bradbury et al., 1998, Guo et al., 1999).

1.3.8.2. *P*2*X*3 receptors are involved in the response to pain

There have been many studies presenting evidence that *P*2*X*3 receptors are involved in the transduction of pain. Research in mouse has shown that *P*2*X*3 is involved in the response to formalin and capsaicin induced models of pain (Tjølsen et al., 1992). The stimulus in these experiments is an injection of a weak solution of either formalin or capsaicin into the hindpaw of a mouse, a well established model for neurogenic pain (Tjølsen et al., 1992).

There are two main phases of nociceptive responses to these painful stimuli: primary neurogenic pain caused by the activation of primary afferent fibres and then a secondary inflammatory response. Because the formalin and capsaicin tests provide a long term nociceptive response that involves a wide range of endogenous pain regulatory substances, it is prefered over the tail-flick test (Dubuisson and Dennis, 1977, Tjølsen et al., 1992). Other studies have shown that injection of ATP into the hindpaw of rats also provides a model for neurogenic pain (Hamilton et al., 1999). After application of the ATP the rat showed intermittent lifting, licking and biting as a behavioural response.

Over-stimulation of the receptor with α,β meATP or inhibition with antagonists such as PPADS or TNP-ATP blocks both the primary and secondary response to pain induced by these tests (Tsuda et al., 1999). An increase in the level of *P*2*X*3 expression in sensory nerve terminals has been reported following nerve damage, another example of ATP acting as a physiological noxious stimuli on *P*2*X*3 receptors (Kennedy and Leff, 1995).

The study of the role of the *P*2*X*3 receptor in pain transduction has been greatly helped by the generation of a *P*2*X*3 knockout mouse (Cockayne et al., 2000). The mouse shows a loss of the rapidly desensitising ATP-induced currents in the dorsal root ganglion cells. Currents recorded from the nodose
ganglion were reduced when compared to wild-type siblings. Antibody staining for IB4 was normal, showing that the cells themselves were unaffected, only the P2X3 receptors had been removed. Injections of ATP into the hindpaw of knockout animals provoked a response that was 77% smaller than that of the wild-type animals.

Studies on mice lacking the capsaicin receptor, VR1, report that the mice show impaired pain perception and nociception, suggesting that VR1 also transduces some of the response to stimulation of the pain pathway (Caterina et al., 2000). This is not surprising for two reasons—firstly both P2X3 and VR1 are found co-localised on the same cells and secondly there are a large number of transmitters involved in the pain pathway.

1.3.8.3. P2X3 receptors on sensory nerve terminals are heteromultimers

Discrepancies between recordings from sensory cells of the rat dorsal root ganglion and recordings from P2X3 receptors expressed in *Xenopus* oocytes lead to the discovery that, in sensory nerve cells, P2X3 receptors act as heteromultimers with P2X2 receptors (Lewis et al., 1995). Application of α,β MeATP to the DRG receptor in vivo produced ATP activated currents that were halfway between those of P2X2 and P2X3 homomultimers when recorded separately. α,β meATP has no activity at the P2X3 receptor and produces fast rising, rapidly desensitising currents at the P2X3 receptor. The receptor native to the rat DRG produces a current which is slower to rise and does not desensitise on application of α,β meATP. This finding gave the first evidence that P2X receptors could act as multimers, forming homo- or heteromultimeric channels in vivo (Lewis et al., 1995). Interestingly, studies on the P2X3 knockout mouse suggest that currents recorded from the DRG are due to homomeric P2X3 receptors, whilst those recorded from the nodose ganglion are due to either homomultimeric P2X2 receptors or heteromultimeric P2X2 and P2X3 receptors (Cockayne et al., 2000). Clearly, different populations of neurons use different receptor combinations to produce the most appropriate
response to agonist stimulation. Other studies have shown that neuron-neuron variation is seen in the DRG of animals. Patch clamp data shows that some neurons express P2X₂ only, some P2X₃ only and others express P2X₂/₃ heteromultimers (Pankratov et al., 2001, Liu et al., 2001). This allows a complex response to ATP application to be seen in the same population of neurons.

1.3.8.4. P2X₃ receptor expression in the enteric nervous system

The enteric nervous system has also been shown to have high levels of P2X₃ receptor expression. Antibodies against P2X₃ receptors in rat and mouse have shown expression on lumbrosacral afferents (also called pelvic afferents) of the bladder. Once again, analysis of the P2X₃ knockout mouse has been instrumental in the elucidation of the role of this receptor in normal bladder function. Studies on human bladder tissue have also shown expression of both P2X₃ and VR1 on nerve fibres scattered throughout the suburothelium (Yiangou et al., 2001).

1.3.8.5. P2X₃ receptors expressed in myelinated pelvic afferents

There are two types of pelvic afferents, with different functions in the bladder. Small myelinated fibres are involved in the non-painful stimulation of the bladder and the natural micturation reflex (Mallory et al., 1989). Larger, unmyelinated fibres are active after painful, pathological distension of the bladder (Häbler et al., 1990). P2X₃ receptors are found to be localised to the smaller, myelinated Aδ fibres, suggesting the receptor has a role in the bladder emptying reflex loop. It has been speculated that distension of all "hollow" organs, such as the lung, ureter and bladder causes a release of ATP (Burnstock, 1999). Recent studies on the bladder of the rabbit and mouse have shown this to be the case (Ferguson et al., 1997, Vlaskovska et al., 2001). This finding allows a model of enteric nervous system control of the bladder to be proposed. Stretching of the mucosa by an increase of volume inside of the bladder causes ATP release. The ATP activates P2X₃ receptors located on the
pelvic afferents of the bladder wall (urothelium) and a voiding reflex is induced. Following micturation, the volume inside the bladder is reduced.

1.3.8.6. Hyporeflexia in P2X3 knockout mice

The P2X3 knockout mouse shows urinary bladder hyporeflexia, a decreased bladder voiding frequency and an increased voiding volume. The response to ATP is significantly delayed and the threshold for receptor activation is much higher. Both the P2X3 knockout mouse and its wild-type litter mates show similar levels of ATP release after stretching. A decrease in the levels of agonist in the distended bladder cannot account for the changes that are seen (Cockayne et al., 1999, Vlaskovska et al., 2000). Antibody staining against the receptor shows normal levels in P2X3+/− mice, whereas no expression was seen in the urothelium of P2X3−/− animals (Cockayne et al., 2000, Vlaskovska et al., 2001). Staining for CGRP and VR1 in the knockouts is unchanged, showing that once again the underlying cell type is still present. The increased voiding volume may well be accounted for by the fact that the receptors have a larger threshold of stimulation before the reflex is initiated (Vlaskovska et al., 2001a).

1.3.8.7. Models of inflammatory disease

Mouse models of inflammatory bowel disease have also been used to further study P2X3 expression levels in the enteric nervous system. The number of P2X3 immunoreactive neurons was significantly increased in the myenteric plexus of inflamed colon compared to controls (Yiangou et al., 2001b).

An interesting result of the work on this receptor has been the finding that smooth muscle samples from human patients with idiopathic detrusor instability (IDI) show a selective loss of P2X3 and P2X6 receptors. The lack of these receptor subtypes may be responsible for the impairment to the control of bladder contractility and lead to bladder incontinence (Moore et al., 2001).

1.3.8.8. Pharmacology of the zebrafish P2X3 receptor
1. General Introduction

The pharmacology of P2X receptors shows wide variation between different species, making it impossible to predict agonist binding efficiencies and antagonist potencies for new cloned receptors. The zebrafish P2X$_3$ receptor has been independently cloned by another group and expressed as a homomultimer, both in *Xenopus* oocytes and HEK-293 cells, and the pharmacological properties have been described (Boué-Grabot *et al.*, 2000, Egan *et al.*, 2000).

Zebrafish P2X$_3$ has an agonist profile that is closest to that of P2X$_7$ receptors, with the largest response being seen after activation by benzoylbenzoyl ATP (bzATP). The next largest response is to ATP and ADP and then $\alpha,\beta$ meATP (bzATP $\gg$ ATP = ADP $>\alpha,\beta$ meATP). The channel shows the fastest activation time and kinetics of desensitisation of any P2X receptor studied so far. After stimulation with an agonist, the channel does not fully recover, even after several minutes of agonist washout (Boué-Grabot *et al.*, 2000, Egan *et al.*, 2000). This is similar to pharmacological recordings from P2X$_1$ receptors (Lê *et al.*, 1999). The P2X$_3$ receptor has been shown to be affected by the application of H$^+$ and Zn$^{2+}$ ions, although the changes in response to agonist application are small. Other receptors such as P2X$_1$ or P2X$_4$ show larger changes in response after ion application (Wildman *et al.*, 1999).

The receptor is highly sensitive to blockade by antagonists. In one study the most potent antagonist was found to be trinitrophenyl ATP (TNP-ATP, Boué-Grabot *et al.*, 2000), whereas a second study only found it to be a potent antagonist at concentrations above 3nm (Egan *et al.*, 2000). However, blockade by the non-competitive antagonist pyridoxal phosphate-6-azophenyl-2', 4' -disulphonic acid (PPADS) is only weak. Suramin also has weak activity (Boué-Grabot *et al.*, 2000).
1. General Introduction

The responses of agonists and antagonists applied to the zebrafish P2X3 receptor place it in a group of P2X receptors that show fast desensitisation, high sensitivity to ATP and α,β meATP and strong inhibition by TNP-ATP (Virginio et al., 1998). Activation of the channel evokes a non-selective inward cationic current. The major responses seen after opening of the P2X3 channel are likely to be caused by the mobilisation of internal Ca2+ stores (Boué-Grabot et al., 2000). One mechanism for this release of Ca2+ could be via the opening of Ca2+ permeable pores on the endoplasmic reticulum.

1.3.9. P2X4 receptor

The zebrafish P2X4 receptor was characterised as part of this thesis. The tissue distribution and a discussion of possible roles of the receptor during development are presented in chapter 4. As a result of this, a more detailed report of the pharmacology and tissue distribution of the P2X4 receptor in other species will be given here.

1.3.9.1 Agonists

The P2X4 receptor forms homomeric channels that are weakly desensitising. Any blocking of receptor activation by antagonist application tends to be easily reversible. The P2X4 receptor is potently activated by ATP followed by ATPγS, 2meSATP, then ADP and α,β meATP: ATP > ATPγS > 2MeSATP >> ADP > α,β meATP (Bo et al., 1995, Seguela et al., 1996). Agonist potencies have been measured from recombinant rat receptors expressed in Xenopus oocytes. Application of ivermectin to the receptor increases the response seen to all agonists (Khakh et al., 1999, rat receptor). Ivermectin is known to allosterically interact with a variety of other receptors including GABA_A and nicotinic α7 receptors. The allosteric regulation of the P2X4 receptor is fully reversible-washing the ivermectin off will restore normal function (Khakh et al., 1999). Another potentiator, cibacron blue, has also been shown to allosterically increase the responses of P2X4 receptors expressed in human embryonic kidney (HEK) cells (Miller et al., 1998). Addition of a general anaesthetic,
propofol (6-diisopropylphenol) to the bath medium of patch-clamp experiments in HEK cells has been shown to cause a potentiation of response after agonist application (Tomioka et al., 2000). Other P2X receptor subtypes were unaffected by the application of propofol. The response seen in P2X4 receptors is dose dependent. All the pharmacological properties of the P2X4 receptor are species dependant - for example, potentiation by ivermectin and suramin has only been detected with the rat P2X4 homologue.

1.3.9.2. Antagonists

P2X4 receptors are usually insensitive to application of known P2 antagonists. Large concentrations of both PPADS and suramin are needed to block channel function (North and Surprenant., 2000). This lack of sensitivity to antagonists is a unique feature of the P2X4 receptor. The reduced sensitivity to blocking has been shown to be due to a mutation in an amino acid at position 249 (Buell et al., 1996). Replacement of the glutamine residue with a lysine restores the ability of antagonists to block the receptor. Human and mouse P2X4 receptors are more sensitive to block than rat receptors. In humans this difference has been linked to a 22 amino acid sequence in the extracellular domain of the receptor (Garcia-Guzman et al., 1997). Mouse P2X4 channels are potentiated by suramin, reactive blue2 and PPADS at low concentrations. At higher concentrations (over 3µm) the channel is blocked by any of these compounds. Comparison of different P2X4 orthologues show that the pharmacological properties of the receptor differ between species. Comparison of agonist binding abilities and the potency of P2X4 antagonists show that the human and mouse receptors have closer related pharmacologies than the rat receptor (Jones et al., 2000). Antagonist potency at the P2X4 receptor has been shown to be species variable.

Extracellular pH can be changed by the release of H+ ions causing acidosis. Localised metabolic acidosis has been associated with several diseases, including bone fracture, ischaemia, inflammation, epileptic seizures
and injuries following damage to the CNS (Chesler, 1990, Wildman et al., 1999a). Lower level transient acidic shifts have also been seen during CNS neurotransmission. Transmitters are packaged in vesicles along with H⁺ ions and release of the transmitter will cause local changes in the pH level (Chesler, 1990, Yanovsky et al., 1995). In parallel, several P2X channels have been shown to have altered pharmacological properties after a change in pH level (King et al., 1996, Stoop et al., 1997). The P2X₂ receptor has been shown to have the highest degree of pH sensitivity. P2X₁, P2X₃ and P2X₄ receptors are also affected but to lesser degrees (Wildman et al., 1999a). The rat and human human P2X₄ receptors have both been studied, the rat homologue appearing to show the greater sensitivity to acidosis (Wildman et al., 1999c, Stoop et al., 1997, Clarke et al., 2000). In contrast to the effects measured at the P2X₂ receptor, H⁺ ions decreased both agonist efficacy and potency at the P2X₄ receptor. A site-directed mutagenesis study showed that the key amino acid mediating this sensitivity to pH level in the human receptor was a histidine residue at position 286. Mutation of histidine to alanine abolished any pH sensitivity (Clarke et al., 2000). The change of pH is thought to be mediated by an allosteric change in the conformation of the channel, affecting its gating ability. The ATP ligand itself is unaffected by the change in pH, suggesting that it is the channel and not the agonist that shows pH sensitivity (Wildman et al., 1997, data from P2X₂ receptor).

The human and rat P2X₄ receptors have been compared and have been shown to have high homology. Histidine 286 is conserved between the two paralogues (Rassendren et al., 1997). Species differences in the size of response following pH modulation is thought to be due to changed channel gating properties. This may be a result of inter-species amino acid changes.

The pH sensitivity of channels may have evolved to facilitate a change in pharmacological properties of receptors following either normal neuronal stimulation or diseases such as ischaemia and hypoxia in the brain (Chesler and
1. General Introduction

Kaila, 1992, Siesjö et al., 1996). The study of the amino acid changes that underlie this pH modulation allows species comparisons and conservation during evolution to be studied.

1.3.9.3. Modulation by metal ions

Both Zn\(^{2+}\) and Ca\(^{2+}\) ions have been shown to potentiate activation of the P2X\(_4\) receptor. Cu\(^{2+}\) ions on the other hand inhibit receptor function, although none of these responses are particularly large compared to those recorded from the P2X\(_2\) receptor (Wildman et al., 1999, Acuna-Castillo et al., 2000). The inhibition by metal ions is non-competitive and ion binding sites have been found in the extracellular domain. Application of other ions, such as cobalt, barium or manganese, did not cause any effect showing that zinc and copper mediated responses were specific (Acuna-Castillo et al., 2000).

1.3.9.4. Tissue distribution of the P2X\(_4\) receptor

The P2X\(_4\) receptor has been found to be expressed throughout the body, with notable expression in the central nervous system, testis and colon. The levels of receptor expression have been shown to change during pregnancy, with diseases such as symptomatic bladder outlet obstruction and in transgenic animals. These changes in expression levels provide evidence that P2X receptors can be synthesised, modified or removed in response to neural signalling. Evidence for P2X\(_4\) tissue expression and function will be discussed separately.

1.3.9.5. Central nervous system

In the central nervous system, expression of P2X\(_4\) has been found in the cerebrum, hippocampus and cerebellum (Rubio and Soto, 2001, Wong et al., 2000, Wang et al., 1996). In the hippocampus, expression was limited to the CA1 region. Expression in the cerebellum was limited to dendritic spines of cerebellar purkinje cells. Immunolabelling for P2X\(_4\) as well as P2X\(_2\) and P2X\(_6\) receptors was found on the postsynaptic membranes of the dendritic spines.
1. General Introduction

(Rubio and Soto, 2001). Patch clamp data from hippocampal granule cells show channel properties that suggest that they may contain a P2X heteromultimer. Possible combinations that would satisfy the results from the patch clamp data include a P2X4/6 heteromultimer or a channel made from P2X1, P2X2, P2X4, and P2X6 subunits (Wong et al., 2000).

Application of ethanol to embryos during development causes abnormalities, and there is evidence to suggest that this may be linked to P2X4 receptor function. P2X4 receptors were expressed in Xenopus oocytes and the responses to ATP application were measured. Following addition of ethanol to the medium containing the Xenopus oocyte, a decrease in the response to ATP was observed (Xiong and Weight, 2000). The ethanol changed the ability of the receptor to bind the ligand, only a small dose of ethanol being required to halve the response to ATP (Xiong and Weight, 2000).

1.3.9.6. Other tissues

Outside of the central nervous system, the P2X4 receptor has been found to be present in a large number of diverse tissues. It is not the aim of this section to give an exhaustive description of protein or mRNA localisation of the P2X4 receptor in every tissue type of the body. Instead, several examples will be chosen that highlight the interesting role of the receptor in non-neural tissues. Appropriate references or reviews will be provided for the other tissues.

1.3.9.7. Thyroid

In the thyroid, expression of a wide number of P2X receptors, including P2X4, has been seen in both follicular cells, endothelial cells and vascular smooth muscle tissue of the adult rat (Glass and Burnstock, 2001). However, no expression of any P2X receptor subtype was found in the C cells of the thyroid. The P2X receptors may have a role in controlling the release of hormones from the thyroid (Glass and Burnstock, 2001).
1. General Introduction

1.3.9.8. Bladder

Several sources have reported expression of P2X4 receptors in the bladder. Yunaev et al., (2000) report that during normal function of the rat urinary bladder, very little P2X4 expression is detected in the detrusor muscle. By day 14 of pregnancy, receptor subtypes expressed in the junctional varicosities of the detrusor muscle have changed; P2X4 and P2X6 receptor protein is detected with an increase of expression of over 80% (Yunaev et al., 2000). Conversely, expression of P2X1, P2X2, P2X3 and P2X5 receptor proteins were seen to have dramatically reduced. It is interesting to find examples of P2 receptor subtypes that change their expression levels in response to a change in tissue function. One possibility is that during pregnancy the innervation needed by the detrusor muscle changes, and the body responds by changing the subtype of receptor that can respond to agonist stimulation. Receptors in the body can be thought of as being a plastic, rather than a rigidly defined, population.

Another study of P2X4 receptor expression in the human bladder describes higher expression of the receptor in biopsy tissue taken from bladder of patients suffering from symptomatic bladder outlet obstruction (O'Reilly et al., 2001). This is another example of a change in receptor subtype to respond to changing levels of neural signalling.

1.3.9.9. Heart tissue expresses P2X4

P2X4 receptors are thought to have a role in modulating cardiac contractility (Hu et al., 2001). Transgenic mice were made by coupling the p2x4 gene to a myosin heavy chain promoter and then injecting the transgene into mouse embryos. The cardiac responses in these animals were measured and compared to wild-type animals. Transgenic mice showed an increased cardiac contractility and relaxation, with no sign of hypertrophy or heart failure. Application of agonists to wild-type and P2X4 transgenic mice was also studied. The transgenics showed an increased response to all agonists, suggesting that
the P2X₄ receptor has a role in modulating cardiac contractility (Hu et al., 2001). By studying the function of the P2X₄ receptor in an artificial situation (ie as a transgene coupled to a different promoter), valuable information is gained about normal receptor function.

1.3.9.10. Xenopus oocytes

Xenopus oocytes contain mechanosensitive cation channels, and in a library screen to try and identify a suitable candidate a P2X₄ receptor was cloned (Juranka et al., 2001). The clone was expressed in human embryonic kidney (HEK) cells and the pharmacology of the receptor was determined by classical agonist application and trace recording experiments. RNase protection assays and RT-PCR were used to show that the gene was present in oocyte mRNA. However, an antibody designed against the P2X₄ receptor protein failed to detect any expression in the oocyte. It is likely that the gene is not active in the oocyte and may well form part of the stored pool of maternal masked mRNA that remains untranslated until later on in development (Juranka et al., 2001).

1.3.9.11. Vascular tissue

In the arteries of the adult rat, P2X₄ expression has been seen to vary according to the size of the artery and vascular bed (Lewis and Evans, 2001). Expression was highest in small and medium sized arteries such as the renal and coronary arteries. In larger arteries such as the femoral or cerebral, the expression of the receptor was lower. These arteries were also seen to have expression of the six other P2X subtypes. In a separate study, the smooth muscle layer of the rat mesenteric artery was shown to express P2X₁, P2X₄ and P2X₅ receptors (Lewis and Evans, 2000). Studies on the rabbit aorta have shown that the expression levels of P2X receptors changed following injury to the endothelial cells (Pulvirenti et al., 2000). P2X₄ clusters were found to be expressed at high levels before injury. After balloon injury to endothelial cells, the density of expression increased by around 10-fold. Other P2X receptor
subtypes showed lower expression levels and did not show marked changed after injury to endothelial cells (Pulvirenti et al., 2000).

1.3.9.12. Retina

Studies of mRNA levels in the rat retina have shown that a few P2X receptor subtypes are expressed in retina cells. A subpopulation of bipolar retinal cells have been shown to contain P2X$_3$, P2X$_4$ and P2X$_5$ mRNA, although P2X$_7$ was not found (Wheeler-Schilling et al., 2000). Muller cells of the rat retina have also been seen to contain mRNA for all of the above receptors (Jabs et al., 2000).

1.3.9.13. Expression in other tissues

Other tissues have been reported to show expression of the P2X$_4$ receptor. Some of the data comes from RNA blot analysis and other studies use localisation of the gene or protein product to study receptor distribution (Bo et al., 1995, Wang et al., 1996). These tissues include the testis, colon, adenohypothysis (Tanaka et al., 1996), spinal cord, lung, vas deferens, adrenal gland (Bo et al., 1995), submandibular salivary gland (Buell et al., 1996), stomach, placenta, bone osteoclasts (Naemsch et al., 1999), prostrate (Slater et al., 2000) B lymphocytes and islet cells of the pancreas (Tanaka et al., 1996).

1.3.10. P2X$_5$ receptor

P2X$_5$ receptor expression has been detected in a wide variety of tissues including lymphocytes, adrenal medulla, the mesencephalic nucleus of the trigeminal ganglia in the brain, the heart, kidney (Cox et al., 2001) and the spinal cord. It was first cloned from the rat coelic ganglion (Collo et al., 1996). The receptor is activated by ATP and 2MeSATP, although $\alpha,\beta$ Me-ATP is inactive. Work on the development of rat and chick skeletal muscle suggests that the P2X$_5$ receptor may have a role in myotube formation (Meyer et al., 1999, Ryten et al., 2001) and expression is also seen in the developing chick heart, brain,
somites, branchial arches and otic vesicle (Ruppelt et al., 2001). Protein expression has been detected in differentiating non-keratinised epithelia and in growing hair follicles (Gröschel-Stewart et al., 1999, Bardini et al., 2000).

1.3.11. **P2X6 receptor**

The P2X6 receptor was cloned from a rat superior cervical ganglion cDNA library (Collo et al., 1996). It is expressed in the developing skeletal muscle of chick (Meyer et al., 1999). P2X6 mRNA is heavily expressed in many areas of the CNS (including the spinal cord and the trigeminal, coeliac and dorsal root ganglia) as well as in epithelial cells of the thymus (Glass et al., 2000). In a similar way to P2X5, the receptor is unaffected by α,β Me-ATP, but is activated by ATP and 2MeSATP.

1.3.12. **P2X7 receptor**

The P2X7 receptor (originally called P2Z) was cloned from both rat macrophages and brain (Surprenant et al., 1996) and has been found to be expressed in bone marrow, hippocampus, cerebellum, (Kim et al., 2001) medulla oblongata, spinal cord (Deuchars et al., 2001) as well as in macrophages, fibroblasts, erythrocytes, lymphocytes and erythroleukemia cells. P2X7 appeared to be expressed as a multimeric complex in bone marrow, but only as a monomer in brain cells (Kim et al., 2001). It has a long intracellular carboxyl terminal and is unique amongst P2X receptors in that its function varies with changes in physiological conditions. Under normal conditions, the channel opens in response to ATP binding and allows small ions to pass through. In the continued presence of ATP and following the removal of divalent cations from the physiological solution the channel properties change and the pore allows small molecules to pass through as well as ions. These small molecules may be up to 900 Daltons in size (Virginio et al., 1997, Coutinho-Silva and Persechini, 1997). This effect is associated with cytotoxicity. P2X7 mRNA and protein has been detected in a wide variety of tissues including granulocytes, monocytes, macrophages and B lymphocytes (Collo et al., 1996). Activation of the receptor
on human macrophages triggers the release of inflammatory cytokines, IL-1β and IL-6 (Ferrari et al., 1997). The most potent agonist at P2X<sub>7</sub> receptors is 3'-O-(4-benzoyl) ATP (benzoyl ATP / BzATP) followed by ATP, 2MeSATP, ATPγS and ADP. Once again α,β-Me-ATP is inactive. P2X<sub>7</sub> receptors are known to be blocked by both isoquinolines and Brilliant Blue G (Jiang et al., 2000, Humphreys et al., 1998). These blocking agents are selective and allow P2X<sub>7</sub> receptors to be distinguished from the P2X<sub>4</sub> subtype—these receptors are often expressed in the same tissues. The functional role of the P2X<sub>7</sub> receptor has been well documented, and it has been linked to cell swelling, vacuolisation and apoptosis (Dubyak and el Moattassim, 1993, Ferrari et al., 1997). Receptor expression has also been seen in the loss of cells from keratinised epithelium, another form of apoptosis (Gröschel-Stewart et al., 1999).

The expression and function of P2X<sub>3</sub> and P2X<sub>4</sub> receptors during zebrafish development will be described in chapters 3 and 4 of this thesis.

1.3.13. P2Y receptors

P2Y receptors are G protein coupled receptors with seven transmembrane domains. The amino terminal of the receptor is extracellular and is thought to have no role in ligand binding. In contrast, the carboxyl terminal is intracellular and is believed to influence signal transduction. P2Y receptors are part of the rhodopsin superfamily of heptahelical G protein coupled receptors.

G protein coupled receptors are present in many tissues of the body and are activated by a wide range of hormones, neurotransmitters and chemoattractants. The stimuli produce their effects by the activation of a signalling cascade involving the action of a G protein second messenger. G proteins are trimeric GTP-binding regulatory proteins. Signalling through a G protein coupled receptor specifically activates one of at least 20 different trimeric G proteins. Binding of the agonist to the receptor causes a replacement of GDP by GTP bound to the α subunit of the G protein. This activation leads to the
dissociation of the GTP bound α subunit from the β and γ subunits. The α subunit proceeds to interact with downstream enzymes or ion channels (Hepler and Gilman, 1992).

The P2Y family of receptors range from 308 to 377 amino acids long and show more divergence in sequence than any other G protein receptor family. Alignment of the human subtypes of P2Y receptor, for example, shows that they have a homology of amino acid sequence ranging between 28-62% identical (King et al., 2000, IUPHAR report). The binding of the ATP ligand to the receptor has been shown to be regulated by a small number of amino acids in the transmembrane domains. Mutagenesis of highly conserved positively charged amino acids in transmembrane domains 3, 6 and 7 significantly reduces the affinity of ATP binding (Jacobson et al., 1999). The majority of P2Y receptors couple via a G protein to activate phospholipase C (PLC), usually PLCβ. Phospholipase C catalyses the hydrolysis of phosphatidylinositol(4,5)-bisphosphate (PtdIns(4,5)-P₂) to the intracellular Ca²⁺ mobilising messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) - the phosphoinositide pathway. Inhibition of adenylate cyclase can also occur in a small number of receptors (North and Barnard, 1997). Activation of IP₃ causes an increase in the intracellular levels of free calcium ions (Ca²⁺). When coupled with DAG, this can in turn activate certain forms of protein kinase C (PKC). This pathway usually utilises a Gq protein to stimulate PLC (Fig. 1.3.). P2Y receptors have been reported to be able to reduce the levels of intracellular cyclic AMP (cAMP) in a number of target tissues (Boyer et al., 2000). The most likely mechanism for this decrease in cAMP level would be inhibition of the enzyme adenylate cyclase by a Gi protein, a Ca²⁺ independent process.

Another second messenger system has been shown to be activated by P2Y₁ and P2Y₂ receptors in rat astrocytes. Binding of ATP to these receptors activates mitogen activated protein (MAP) kinases (Neary and Zhu, 1994) in a
Figure 1.3. Diagram of a P2Y receptor showing the signalling pathway used by most of the receptor subtypes. See text for full explanation.
pathway that has been shown to be separate from the RAF kinase pathway. The signalling from P2Y receptors is slower than that of P2X receptors and is more suited to modulatory roles for ATP and its analogues than to signalling at synapses.

1.3.14. **P2Y receptor orphans**

P2Y receptor like "orphans" also exist. These are structurally related heptahelical proteins that are extremely similar to P2Y receptors and are considered to be part of the same G protein coupled receptor subfamily (Marchese et al., 1999). These recombinant receptors are monomers and their association with P2Y receptors has been questioned. Of a total of 12 receptors found in avians, amphibians and mammals, there are currently six cloned mammalian P2Y receptors: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11 and P2Y12. Lower case letters (i.e. p2y) are used for receptors that have not yet been shown to respond to P2 receptor agonists. p2y5, p2y7, p2y9 and p2y10 were first thought to be family members but later turned out to be unresponsive to ATP when expressed in cell lines. The p2y3 receptor is now known to be the homologue of human p2y6 and p2y7 has been reclassified as a leukotriene B4 receptor. (Ralevic and Burnstock, 1998, King et al., 2000). p2y9 was cloned from a *Xenopus* cDNA library and has been shown to be abundantly expressed in developing *Xenopus* embryos. It is activated by all five naturally occurring nucleoside triphosphates (ATP, UTP, GTP, CTP and ITP) to a similar degree, resulting a biphasic Ca^{2+} dependent Cl⁻ current. The receptor has not been found to have any mammalian homologue (Bogdanov et al., 1997).

1.3.15. **P2Y1 receptor**

P2Y1 is perhaps the most developmentally interesting of the P2Y family of receptors to have been found to date. It has been shown to be expressed in the developing wing bud of chick embryos, as well as mesonephros, brain, somites and facial primordia (Meyer et al., 1999). The P2Y1 receptor does not appear to play a role in the development of the central nervous system or somites. In the
1. General Introduction

developing limb buds, from developmental stage 19 onwards, P2Y1 is thought to cause apoptosis between digits to facilitate correct limb bud patterning. Strong expression of the gene was seen in the areas where cartilage would form. Expression has also been seen in human placenta, heart, blood platelets, skeletal muscle, pancreas, prostrate, ovary and intestine. Another major functional role of P2Y1 is the induction of aggregation in platelets. Activation of the receptors causes a change in the shape of the platelets, an increase in intracellular calcium ions and aggregation. P2Y1 deficient mice show an increased bleeding time, in accordance with the role of P2Y1 in aggregation (Fabre et al., 1999, Leon et al., 1999). P2Y12 receptors were also found to have a role in aggregation of platelets: this will be discussed later in this introduction. P2Y1 receptors are activated by adenine but not uridine nucleotides (i.e. ADP and ATP instead of UDP and UTP). ADP is the most potent of these agonists and it is not yet known whether ATP acts as a full or a partial agonist. There are some reports that ATP can act as a full antagonist at the P2Y1 receptor and that agonist effects are only seen after enzymatic degradation from ATP to ADP (Sak et al., 2000). The P2Y1 receptor is also able to be activated by synthetic agonists and antagonists, including compounds such as 2me-SADP (a potent agonist) and 2-meSATP. The downstream target of the receptor is a Gq/11 protein which in turn activates PLC.

1.3.16. P2Y2 receptor

The P2Y2 receptor has not been reported to have a developmental role. It has been shown to be expressed in a variety of cell types-including bovine aortic endothelial cells (BAEC) and human erythroleukemia cells (Pirotton et al., 1999, Baltensperger et al., 1997). Expression in rats has been seen in the intestines, gallbladder and airways (Cressman et al., 1999). In humans, expression has been seen in the brain, heart, lung, kidney, placenta and skeletal muscle. P2Y2 is activated with equal potency by ATP and UTP but is insensitive to both ADP and UDP. For this reason the receptor was originally called the P2U receptor. There are no selective antagonists of P2Y2, however suramin
1. General Introduction

acts as a potent non-selective antagonist. The signalling mechanisms downstream from P2Y2 appear to change according to the tissue in which the receptor is expressed. Both Gι0 and Gq/11 proteins are used as second messengers. In airway epithelial cells PLC is activated along with mobilisation of internal Ca2+ ions. Ca2+ sensitive Cl− ion channels on the airway epithelial cells open and fluid is secreted to line the walls of the airways (see Cressman et al., 1999). This signalling pathway may be manipulated to treat cystic fibrosis, a disease characterised by a failure of ion channels to secrete Cl− ions into the airways. Agonists of the P2Y2 receptor (including UTP) are being investigated pharmacologically to be used as treatments for cystic fibrosis (Stutts et al., 1992, Parr et al., 1994, Cressman et al., 1999). P2Y2 receptor agonists may also be useful in the treatment of other diseases that affect secretion of ions across membranes. Examples of this include dry eye and chronic bronchitis.

1.3.17. P2Y4 receptor

The P2Y4 receptor has a pharmacological profile that is very similar to that of the P2Y2 receptor. The rat P2Y2 receptor is activated with equal potency by ATP and UTP and is not affected by either of the nucleoside diphosphates (ADP and UDP). The receptor was first cloned from rat heart and is antagonised by PPADS and Reactive blue 2 but not suramin (Charlton et al., 1996). The inability of suramin to antagonise P2Y4 allows the pharmacological differentiation between P2Y4 and P2Y2 receptors (Bogdanov et al., 1998). In rat tissue, P2Y4 receptors may be responsible for some of the responses to agonists originally attributed to P2Y2 receptors. mRNA has been found to be expressed in rat brain, heart, pancreas and astrocytes. P2Y4 mRNA expression has also been found in human placenta and in mouse liver, intestine, stomach, bladder and lung (Suarez-Huerta et al., 2001).

1.3.18. P2Y6 receptor

The P2Y6 receptor was cloned by a low stringency screen of a human placenta library using a probe made against the P2Y4 receptor. P2Y6 is
expressed in human spleen, thymus, bone, heart, kidney, stomach placenta and intestine (Communi et al., 1996). It is activated most potently by UDP, with nucleotide diphosphates and ATP acting as only weak agonists. The channel is non-selective inhibited by Reactive blue2, PPADS and suramin. The transduction mechanism appears to use a Gq/G11 G protein to activate PLCβ and so elevate intracellular Ca^{2+} levels. A functional role for this receptor has not yet been found. Together with the P2Y4 receptor, P2Y6 forms a subgroup of pyrimidinoreceptors inside the P2Y family.

1.3.19. P2Y_{11} receptor

The zebrafish P2Y_{11} receptor was characterised as part of this thesis. The tissue distribution and a discussion of possible roles of the receptor during development are presented in chapter 5. As a result of this, a more detailed report of the pharmacology and tissue distribution of the P2Y_{11} receptor in other species will be given here.

1.3.19.1 Pharmacology

There have been many studies of the P2Y_{11} receptor in which the human receptor has been expressed in either a Chinese hamster ovary (CHO-K1) or human astrocytoma (1321N1) cell line and agonist and antagonist potencies have been measured. The P2Y_{11} receptor has been shown to be selective for adenine nucleotides. Expression of the channel in different cell lines has lead to slightly altered pharmacological properties being reported in different studies (Communi et al., 1999, Qi et al., 2000). The general consensus is that the agonist potency is ATPγS ≈ BzATP > dATP > ATP > ADPγS > 2MeSATP > ADP. The human P2Y_{11} receptor is strongly inhibited by suramin, less strongly inhibited by reactive blue 2 and is not affected by PPADS. The synthetic antagonist MRS 2179 does not appear to have been tested on P2Y_{11} receptors. Adenosine-3'-phosphate-5'-phosphosulphate (A3P5PS, a bisphosphate derivative) acts as an antagonist (von Kügelgen and Wetter, 2000). P2Y_{11} is slowly desensitised in the presence of continued agonist application. For
1. General Introduction

example, application of dATP to the receptor can cause an increase of intracellular Ca^{2+} levels for periods of up to 30 minutes (Suh et al., 2000). Removal of the dATP caused an immediate decrease of the calcium ion levels.

1.3.19.2. Activation of two signalling pathways

The P2Y_{11} receptor is the only P2Y receptor that has been shown to activate two signalling pathways. Binding of agonists to the receptor have been shown to activate both the phospholipase C (PLC) and adenylate cyclase (AC, causes formation of cAMP) pathways. It is thought that stimulation of PLC occurs via a G_{q} protein, whereas AC is activated by a G_{s} protein (Suh et al., 1998). Initial studies suggested that agonist application activated both pathways with equipotency (Communi et al., 1998, Communi et al., 1997), but later research suggests that the PLC pathway is 15 times more potently activated than the AC pathway (Qi et al., 2001). The discrepancies between the two reports was due to the receptor being expressed in two different cell lines in the first study and then in the same cell line in the second. Stimulation of PLC leads to an increase in intracellular Ca^{2+} ion concentration and an activation of Protein kinase C (PKC). PKC in turn potentiates the formation of cAMP by stimulating the enzyme adenylate cyclase, an example of receptor mediated promotion of signalling (Qi et al., 2001). On the other hand, PKC acts to inhibit further PLC activation. Studies of P2Y_{11} receptors expressed in human promyelocytic leukaemia cells (HL-60 cells) have shown that there is no cross talk between the two signalling pathways (Suh et al., 2000).

Expression of P2Y_{11} receptors in Madin Darby canine kidney (MDCK) null cells (which have all known P2 receptors removed) has also been used to show that the P2Y_{11} receptor can activate two different signalling pathways (Zambon et al., 2001). The P2Y_{11} receptor were found to be localised to the basolateral membrane of MDCK. The increase in cAMP that was measured in the MDCK cells was not inhibited by either cyclooxygenase or BAPTA (Zambon et al., 2001) suggesting that the P2Y_{2} receptor was not involved. The canine receptor
shows 70% homology to the human paralogue. The agonist potency of the canine receptor was similar to that of the human clone, although ATP was found to be a less potent agonist of the canine paralogue (Zambon et al., 2001).

There are only a few examples of receptors that can activate both PLC and AC: these include luteinising hormone, calcitonin, thyrotropin, parathyroid hormone, pituitary adenylate cyclase-activating polypeptide and three tachykinins (Qi et al., 2001). These are all class 1b receptors (Bockaert and Pin, 1999) that recognise peptide hormones as their ligands. Sequence analysis of P2Y receptors shows them to have higher homology to peptide receptors than to receptors for small biogenic amines and adenosine (Lustig et al., 1993), suggesting that this ability to activate both PLC and AC may be restricted to a small class of 1b receptors. Along with the P2Y_{11} receptor, only the tachykinins have the ability to increase the levels of both PLC and AC (Qi et al., 2001). Activation of other receptors causes a decrease in cAMP levels.

1.3.19.3. Molecular characteristics

The human P2Y_{11} receptor was cloned from a placenta library in a low stringency screen using a probe designed against the human P2Y_{4} receptor (Communi et al., 1997). The gene coding for the receptor had an open reading frame of 1113 base-pairs. The protein product is 371 amino acids long. The gene has one intron, which is very unusual for members of the P2Y family (Communi et al., 1997). Other P2Y genes such as P2Y_{1}, P2Y_{2} and P2Y_{6} (Schachter et al., 1996, Janssens et al., 1996, Godecke et al., 1996) have previously been shown to be intronless. The first exon of the P2Y_{11} receptor contains the initiation codon and an N-glycosylation site. The second exon contains all seven transmembrane domains (Communi et al., 2001).

The P2Y_{11} gene is unique in its ability to form intragenic splices with the human SFF1 gene. Both genes are found next to each other on human chromosome 19 (Communi et al., 2001). SFF1 is a homologue of a gene found
in *Saccharomyces cerevisiae* called *suppressor of swi four (ssf)* and which is involved in mating of yeast cells. In *Drosophila* there is a related gene called *ppan* that has been shown to be involved in larval growth (Migeon *et al.*, 1999). Intragenic splicing between genes is very rare and only three examples have been reported in the literature so far. These include *MDS1* and *EVI1*, *Prndp* and *Prnp* murine genes and Galactose-1-phosphate uridytransferase with interleukin-11 receptor α chain gene (Moore *et al.*, 1999, Magrangeas *et al.*, 1998, Fears *et al.*, 1996). The splicing of *P2Y11* and *SSF1* is the first example of intragenic splicing involving a G protein coupled receptor. Splicing of the two genes together causes a loss of the first codon of the *P2Y11* receptor and confers a novel ATP binding site from the *SSF1* gene to the *P2Y11* gene. Expression of the chimaera in a CHO-K1 cell shows that the response to nucleotides in unchanged when compared to CHO-K1 cells that contain the *P2Y11* receptor alone (Communi *et al.*, 2001). The only difference that is reported is that the basal cAMP levels and the maximum accumulation of cAMP are decreased (Communi *et al.*, 2001). The function of the formation of this chimaera is not currently understood. It is possible that the chimaera in vivo has different properties to those measured in CHO-K1 cells. It may provide a way to down-regulate the number of *P2Y11* receptors or may be involved in a completely different process.

1.3.19.4. *Stimulation of renin in human kidney cells*

Following transfection of the human *P2Y11* receptor into Calu-6 cells (a kidney cell line), stimulation with ATP was seen to increase the levels of renin protein (van der Weyden *et al.*, 2000). The *P2Y11* receptor caused phosphorylation of the cAMP response element binding protein (CREB) and so activated the cAMP response element (CRE). This in turn caused an increase in the activation of the renin promoter and so increased transcription of renin protein (called REN in humans, see van der Weyden *et al.*, 2000). One of the main sources of ATP as a ligand for receptors is release following cytolytic damage (see Bodin and Burnstock, 1998 or the introduction to chapter 1). An
1. General Introduction

intriguing possibility is that P2Y₁₁ (which has been detected in cardiac muscle and renal tissue) could be able to play a role in the pathogenesis of cardiomyopathy or renal disease following the cytolytic release of ATP from damaged tissue (van der Weyden et al., 2000).

1.3.19.5. Tissue distribution

Apart from the zebrafish P2Y₁₁ receptor homologue which was characterised as part of this thesis and is described later on in this chapter, only the human P2Y₁₁ receptor has been discovered to date. There have been no in situ reports of receptor expression. All the data so far comes from reverse transcription polymerase chain reaction (rtPCR) experiments to look at receptor distribution. The P2Y₁₁ receptor has been found to be present in foetal brain, kidney, testis, muscle, liver and spleen (van der Weyden et al., 2000) as well as in the small intestine and cells of the immune system (Communi et al., 1997). In HL-60 cells P2Y₁₁ is thought to be involved in granulocyte differentiation to form neutrophils (Jiang et al., 1997).

1.3.19.6. P2Y₁₁ function in HL-60 cells

Human promyelocytic HL-60 leukaemia cells (HL-60 cells) can be induced to differentiate into neutrophil like cells by the addition of various agents to their medium. These agents include histamine, adrenaline and ATP (Jiang et al., 1997) and all have the ability to increase cAMP levels. The mechanism by which ATP was able to induce HL-60 differentiation was unknown for a long time. Initial studies suggested that degradation of ATP to ADP, AMP and finally adenosine would allow activation of A₃ receptors. Adenosine has already been shown to have a role in stimulating apoptosis in HL-60 cells (Tanaka et al., 1994). Analysis of the agonist affinities of the receptor mediating this differentiation in HL-60 cells suggested that a novel P2 receptor was involved (Jiang et al., 1997, Choi and Kim, 1997). The receptor was shown to increase cAMP levels in the cell line by activating both the enzyme adenylate cyclase and protein kinase A. Further work found that the P2Y₁₁ receptor cloned by
Communi et al. (1997) was expressed in HL-60 cells and had a similar agonist profile to the novel P2 receptor previously described as being able to increase differentiation. Application of ATP to HL-60 cells stimulates P2Y₁₁ receptors and causes an increase in cAMP levels. This in turn increases the differentiation of HL-60 cells into neutrophils.

The function of the P2Y₁₁ receptor in the maturation of other cell types has also been studied. Dendritic cells (DC) are antigen producing cells in the immune system that are able to induce primary immune responses (Banchereau and Steinman, 1998). DC precursors migrate to lymphoid tissues where they activate lymphocytes, secrete cytokines and generate immune responses. There have been several studies showing that ATP can synergise with TNFα to cause maturation and activation of DC (see Delneste et al., 1999). ATP has been shown to initiate this maturation by activation of the P2Y₁₁ receptor (Wilkin et al., 2001). Firstly responses to ATP were blocked by suramin suggesting that a P2 receptor was involved and secondly activation of the receptor on DC showed a similar agonist profile to that of the P2Y₁₁ receptor cloned from HL-60 cells (Wilkin et al., 2001).

1.3.19.7. P2Y₁₁ receptors stimulate secretion in pancreatic cells

Pancreatic duct epithelial cells (PDEC) mediate the exocrine secretion of fluids and electrolytes from the pancreatic duct. The secretory function of PDEC results from the stimulation of many ion transport pathways including the cystic fibrosis transmembrane conductance regulator (CFTR), cAMP Cl⁻ channels, Ca²⁺ as well as K⁺ and Na⁺ channels (Nguyen et al., 2001). Furthermore, the presence of a wide variety of P2 receptors (both P2X and P2Y subtypes) has been reported in the literature (Nguyen et al., 1998). ATP has been shown to cause secretion from PDEC by activating either P2Y₂ or P2Y₁₁ receptors (Nguyen et al., 2001, Nguyen et al., 1998). Addition of ATP to cultured canine PDEC was found to cause an increase of cAMP following P2Y₁₁ receptor activation. This effect was not blocked by the addition of BAPTA, a known
calcium chelator, which had previously been shown to block the P2Y2 receptor response in PDEC (Nguyen et al., 1998). Elevated cAMP levels was shown to stimulate the CFTR Cl- channel and mediate secretion from the pancreatic duct. The agonist profile of the canine PDEC expressed receptor was similar to that previously published for the P2Y11 receptor in HL-60 cells (Nguyen et al., 2001, Jiang et al., 1997).

1.3.20. P2Y12 receptor

P2Y12 receptors are found on platelets in a similar way to the P2Y1 receptor (Hollopeter et al., 2001). Activation by ADP causes the inhibition of adenylate cyclase by a G protein and is required for platelet aggregation. The P2Y1 receptor is thought to be important for the initiation of aggregation and the P2Y12 receptor is needed for the completion of aggregation and stabilisation of aggregates (Gachet, 2001). The P2Y12 receptor has also been shown to be the target for antithrombotic drugs such as ticlopidine and clopidogrel (Hollopeter et al., 2001, Barnard and Simon, 2001). Antagonists of the P2Y12 receptor are now being developed to try to treat blood clotting disorders (Storey et al., 2001). Interestingly, a patient with a blood clotting disorder has been diagnosed as having a defect in the gene coding for the P2Y12 receptor. The P2Y12 receptor has a restricted tissue expression. It is found in platelets and at lower levels in the brain and is absent from all other tested tissues. This makes it an extremely good target for antithrombotic drugs.

1.3.21. P2Y13 receptor

The most recently cloned member of the P2Y receptor family is P2Y13. P2Y13 has been cloned from both human and mouse, and shows high sequence similarity to a receptor orphan called GPR86 or SP174 (Communi et al., 2001, Zhang et al., 2002). Together with the P2Y12 receptor, P2Y13 is thought to form a new subgroup of P2Y receptors with structural differences to other family members and high affinity to ADP (Communi et al., 2001). Activation of P2Y13 causes a decrease in adenyl cyclase activity and an increase in MAPK
stimulation. rtPCR analysis and Northern blotting have suggested that the receptor is expressed in the spleen, brain and liver of both humans and mice (Communi et al., 2001, Zhang et al., 2002). A developmental role for the receptor has not yet been reported in the literature.

The expression of a novel P2Y\textsubscript{11} receptor during zebrafish development will be described in Chapter 5 of this thesis.

1.4. Release and removal of ATP during purinergic signalling
1.4.1. Release of ATP into the extracellular space

ATP is a large molecule that carries a charge and as such cannot cross the cell membrane. In order for it to act as an agonist at receptors, it therefore has to be released near to the place where its receptors are found. Research has shown that ADP and UTP can also be released, although most research has concentrated on the release of ATP. A variety of different cell types show ATP release following mechanical stimulation. These include red blood cells, platelets, endothelial cells, chondrocytes and Ehrlich ascites tumour cells (Person et al., 1999, Bodin and Burnstock, 2001a). A good example of this is the release of ATP and ADP from red blood cells (Bozzo et al., 1999). There have been many studies showing that erythrocytes are able to release ATP following a fall in the oxygen content of the blood and as the haemoglobin becomes desaturated. The intraluminal ATP induces a vasodilator response which results in an increase in tissue perfusion of oxygen (Ellsworth, 2000). The release of ADP and ATP at sites where tissue damage has occurred is also thought to be very important in the role of ATP mediated nociception.

In general, there are three main ways in which ATP can be released: vesicular release, release following cytolysis and ATP-binding cassette (ABC) protein release. The intracellular concentration of ATP and its analogues is usually between 2-5mM before it is released extracellularly to act on receptors.
1. General Introduction

Vesicular release is extremely rapid and is important for signalling from nerve terminals. Vesicles containing ATP are also seen in non-neuronal cells. In nerve terminals, the ATP is stored in presynaptic vesicles that are released by exocytosis following nerve stimulation. ATP and ADP is copackaged with conventional neurotransmitters in the vesicles. It was the discovery of NANC transmission, following vesicular release in nerve terminals, that first prompted the proposal that ATP could act as a signalling molecule. The receptors are found postsynaptically and following receptor activation, the ATP is removed by enzymatic breakdown and resorption. The products of the breakdown of ATP (ADP and adenosine for example) can themselves continue and stimulate other receptors. Vascular endothelial cells are also thought to release ATP by vesicular transport (Bodin and Burnstock 2001b).

Cytolysis is the breakdown of cells that follows biological or physiological trauma. The cells either become damaged or apoptose and the intracellular contents are released at the site of the trauma. Following cytolysis, the concentration of nucleotides released has been shown to be as high as 20μM, a level that is sufficient to activate all P2 receptor subtypes (Born and Kratzer, 1984). High ADP levels were also measured, probably due to the action of enzymes that have broken down the ATP at the site of tissue damage. Platelets and leukocytes are also able to release ATP at the site of tissue damage. An example of this was demonstrated by measurements of ATP released from human vascular endothelial cells (HUVECS) following shear stress. This finding shows that ATP can be released from tissues after laboratory stimulated inflammation and suggests that ATP is important in the inflammatory response to tissue damage (Bodin and Burnstock, 1998).

ATP-binding cassette (ABC) proteins are ATP dependent membrane transporters that have two cytoplasmic ATP binding domains and two hydrophobic transmembrane domains (for review, see Dean et al., 2001). The transmembrane domains allow the transport of amino acids, inorganic ions,
sugars and proteins across the cell membrane. The ABC proteins bind ATP and use the energy that is gained to drive the transport of molecules across the membrane. Most of the evidence that ABC proteins can act to increase the levels of ATP at receptors comes from the use of specific ABC protein blockers. ATP release from Ehrlich ascites tumour cells is inhibited by the addition of glibenclamide, a blocker of the cystic fibrosis conductance regulator (CFTR). Glibenclamide is a sulphonylurea that is thought to be a blocker of a wide variety of ABC proteins (Payen et al., 2001). There are three common ABC proteins that have been associated with ATP transport: CFTR receptors, the sulphonylurea receptor and P-glycoprotein, which is the product of transcription of the multidrug resistance protein gene (Jedlitschky et al., 2000).

1.4.2. Breakdown and removal of ATP

The first step for the removal of ATP from receptor binding sites is enzymatic breakdown by cell-surface located enzymes that sequentially strip ATP of its phosphates. The 5'-di and 5'-monophosphates, free phosphates and pyrophosphate that are made by this process are then removed from the extracellular fluid and can be recycled by neighbouring cells. Many of the products formed during this degradation can act on other receptor types. UDP, for example, has been shown to act on the P2Y6 receptor (Chambers et al., 2000). Adenosine, the final product in the ectonucleosidase chain that degrades ATP, is able to act on P1 receptors in the vicinity and so mediate further responses in the body. In the general hydrolysis cascade, seen in both in vitro and in vivo measurements, ATP is sequentially degraded to ADP, AMP and finally adenosine.

There are four main families of enzymes that are capable to do this: alkaline phosphatases, ecto-5'-nucleotidases, the E-NTPases (also called E-ATPases or apyrases) and phosphodiesterase/pyrophosphatases (the E-NPPs family). The overall sequence of events and structure of the cascade is complicated and not fully understood. There have been no studies looking at
either the functional diversity or redundancy of these enzymes, work which would greatly benefit from the generation of knock-out mice for one of the enzymes (for review see Zimmermann, 2000). Several mice with mutations in genes coding for ectonucleosidases have been generated, although their phenotypes are less severe than would have been predicted (Enjyoji et al., 1999, MacGregor et al., 1995). The study of these ecto-enzymes is further complicated by the fact that they are targets for some P2 antagonists, such as suramin (Heine et al., 1999). There is a lot of research needed before the whole process of ATP degradation and removal is well characterised. The rapid degradation of ATP and its analogues has made pharmacological characterisation of P2 receptors difficult. A single tissue type may express receptors activated by UTP, UDP, ADP and adenosine as well as ATP.

1.5 P2 receptors during development

P2 receptors have been shown to be expressed during both the embryonic and postnatal development of many tissues (for review, see Burnstock, 2001b). The number and diversity of organ systems that show expression is almost overwhelming, although functional studies are not so common. P2 receptors have been seen to regulate a broad range of responses in adult tissues and cells which suggests that they could have a role during embryonic development. ATP has been seen to regulate both short term signalling events in the nervous system (such as endocrine and exocrine secretion) and longer term changes including cell proliferation and differentiation. These processes are important during the repair and regeneration of adult tissues following trauma and during embryonic development. Many of the functions of ATP and its analogues during development can be attributed to its ability to increase the concentration of intracellular Ca^{2+} ions. Ca^{2+} is known to have multiple diverse actions during development, making it probable that P2 signalling will also be found to be developmentally important.
1. General Introduction

1.5.1. P2 receptors can regulate cell growth and differentiation

The mitogenic activities of purine nucleotides have been reported in a wide variety of cell types, including Swiss mouse 3T3 and 3T6 fibroblasts, endothelial cells, vascular smooth muscle, primary astrocytes and astrocytoma cell lines (Burnstock, 2002, Chung et al., 1997). Application of UTP and ATP to C(6) glioma cells caused MAPK activation and a time and dose dependent incorporation of (3)H-thymidine. This confirmed that activation of P2Y<sub>2</sub> receptors has a mitogenic effect (Tu et al., 2000).

A good example of this are the purine nucleotides that have been seen to play a role in the glial response to tissue damage. Astrocytes are non-neuronal support cells found in the central nervous system. ATP is known to act as both a mitogenic and a morphogenic agent. Stimulation of P2Y receptors has been found to increase the level of DNA synthesis in astrocytes, neuroblastoma and astrocytoma cells, (Neary and Zhu 1994). P2X receptors have not yet been found to be involved in this process. The P2Y receptors are thought to be connected to the MAP kinase pathway, key signal transducing pathways involved in cellular proliferation, differentiation and responses to stress. Application of ATP to astrocytes has been shown to cause a rapid stimulation of MAP kinase activity. Blocking with P2, but not P1, antagonists shows a reduction in the response to ATP. ATP has been found to act synergistically with members of the fibroblast growth factor (FGF) family when inducing DNA synthesis in astrocytes (Neary et al., 1994). The application of ATP or UTP to the astrocytes leads to the formation of activator protein (AP1) complexes, which are transcriptional activators implicated in the expression of a diverse number of genes (John et al., 2001, Neary et al., 1996). The formation of the AP1 complex may well be responsible for the trophic activity of ATP on astrocytes. FGF family members, on the other hand, signal via the Ras/Raf pathway. This divergence in downstream pathways used during mitogenesis explains the synergistic enhancement of ATP and FGF family members acting together. The P2 receptor response in astrocytes may have a role in the gliotic response to
brain injury. Following tissue damage, ATP is released and stimulates DNA synthesis, leading to proliferation and morphological responses.

Mitogenesis following extracellular nucleotide release has also been observed in vascular smooth muscle cells (VSMCs). Studies that looked at VSMCs in culture, following the addition of ATP or UTP, found that activation of a P2Y$_2$/P2Y$_4$ receptor resulted in an increase of the intracellular Ca$^{2+}$ ion concentration and a subsequent transition of the cell from the G$_1$ to the S and M phases of the cell cycle. This in turn leads to DNA synthesis (Miyagi et al., 1996). The receptors had no effect on progression from stage G$_0$ to stage G$_1$ of the cell cycle. ATP is also able to induce proliferation of VSMCs by using a similar pathway to that used in astrocytes. Activation of a P2Y receptor by ATP leads to PLC formation, an increase in the Ca$^{2+}$ ion concentration and a stimulation of PKC, MAP kinase and Raf1.

1.5.2. P2 receptors can regulate apoptosis during development

Several studies have suggested that ATP can act as a determinant to switch between necrosis and apoptosis of cells (Eguchi et al., 1997). In contrast to necrosis, apoptosis is a highly regulated process that follows well defined biochemical stages. DNA in cells is condensed towards the inner nuclear membrane and is then systematically degraded into small fragments. Necrosis, on the other hand is not such a rigidly defined process that is characterised by mitochondrial swelling and loss of the integrity of the plasma membrane. Apoptosis tends to be carefully regulated; necrosis often occurs accidentally. A lack of ATP will cause a cell to undergo necrosis. The presence of ATP, on the other hand, will activate caspases and necrosis will occur (Ferrari et al., 1999).

There are two members of the P2X receptor family that have been implicated in apoptosis: P2X$_1$ and P2X$_7$. P2X$_1$ shows high sequence homology to the RP-2 gene and P2X$_1$ mRNA has been seen to increase during the apoptosis of thymocyte cells (Valera et al., 1994, Valera et al., 1995, Chvatchko...
1. General Introduction

et al., 1996). The P2X_7 receptor is activated by ATP^4- and has the unique property that it is able to form a pore that allows large molecules of >900 Daltons to pass through as well as acting as being a selective cation channel (Surprenant et al., 1996). The P2X_7 receptor has been seen to be involved in both necrosis in macrophages and in apoptosis in microglia (Chiozzi et al., 1996, Ferrari et al., 1997). Several studies have also shown that P2 receptor antagonists can provide protection to populations of neurons that would normally undergo apoptosis (Cavaliere et al., 2001a, Cavaliere et al., 2001b)

1.5.3. Early roles for nucleotides in embryogenesis

As the discussion above shows, studies of nucleotide receptors found to be active during the repair and growth of adult cells suggests that they may well have a role in similar processes during embryonic development. There is not a lot of evidence for P2 signalling during embryogenesis, and this section will briefly discuss the findings so far.

Extracellular ATP is needed for the acrosomal activation of the human spermatazoa (Foresta et al., 1992) and an ATP activated Na^+ channel is involved in sperm induced fertilisation (Kupitz and Atlas, 1993). These pieces of evidence suggest that ATP has an important role in the initiation of development in oocytes. ATP activated receptors have also been seen to act in concert with muscarinic receptors to increase the levels of IP_3 and intracellular Ca^{2+} ions in cells of the gastrulating chick embryo (Laasberg, 1990).

P2 receptors signalling during embryonic development will be discussed in detail below. The two best characterised receptors during embryonic development are chick P2Y_1 and Xenopus P2Y_8. These receptors will be discussed first.

1.5.4. Chick P2Y_1
One of the best characterised gene expression patterns for this receptor family is the \( p2y_1 \) gene in chick (Meyer et al., 1999). \( cP2Y_1 \) has been shown to be expressed in the telencephalon, dorsal diencephalon, posterior midbrain and also in a small area of the anterior hindbrain that may correspond to the trochlear motor nucleus. The expression is first seen at around chick developmental stage 33 and increases in intensity by stage 38. Expression in the dorsal diencephalon is predominantly restricted to the grey matter and is not seen in the ventral zone cells. \( cP2Y_1 \) does not have an early expression pattern and so is unlikely to have an early role during chick development. Other species show different areas of expression for the \( P2Y_1 \) receptor in the brain. In humans, at the cellular level, the \( P2Y_1 \) receptor was strikingly localised to neuronal structures of the cerebral cortex, cerebellar cortex, hippocampus, caudate nucleus, putamen, globus pallidus, subthalamic nucleus, red nucleus, and midbrain (Moore et al., 2000). \( P2Y_1 \) expression was also seen in other developing organs in the chick. These will be discussed later in the introduction.

1.5.5 Frog \( P2Y_8 \)

\( p2y_8 \) was cloned from a \( Xenopus \) cDNA library and shows transient expression in the tailbud and neural tube at a time which is coincident with a role during neurogenesis. The gene was first detected at \( Xenopus \) developmental stage 13-14 when an arc of expression is seen in the anterior neural plate. The expression spreads to fill all the tissue of the neural plate and then reduces until it is only detected in the neural tube that forms near the tailbud by stage 28 (Bogadanov et al., 1997). The onset of \( Xenopus \) \( P2Y_8 \) expression during late stages of gastrulation suggests that it is not involved in the initial induction of the neural plate but is an early response to signals from the plate tissue. It may well be important for secondary neuralising signals. Following activation by a nucleoside diphosphate, a prolonged cellular response is seen (lasting up to 40-60 minutes) and this includes an increase in the levels of \( Ca^{2+} \) in the embryo. Increases in \( Ca^{2+} \) ions are known to regulate cell proliferation, migration and differentiation, all processes known to be important during development. The
precise role that *Xenopus* P2Y\textsubscript{8} plays during *Xenopus* neural plate formation is not yet known.

1.5.6. *Limb bud development*

Chick P2Y\textsubscript{1} has been found to be expressed in the developing limb buds. *In situ* hybridisations found P2Y\textsubscript{1} to be expressed in the posterior third of stage 19 chick embryo wing buds (Meyer *et al.*, 1999). By stage 21 the expression spread to all parts of the wings and limbs, apart from the anterior third sections of both. Sections through the limbs showed asymmetric expression, dorsal expression being stronger than ventral. By stage 25, a uniform ring of P2Y\textsubscript{1} expression was seen around the core of the limb bud. At the time of digit formation, P2Y\textsubscript{1} expression was present in all digits, the strongest expression being found in the most posterior digit. The expression was absent from ectoderm, cartilage, tendons and joint forming areas, but was present in limb bud mesenchyme. Expression in the mesenchyme was dynamic and varied according to both stage and position within the limb.

1.5.7. *Retina development*

Adenosine and ATP have both been shown to have a role in the developing retina, their abilities to increase Ca\textsuperscript{2+} ion expression making them important signalling molecules. The Ca\textsuperscript{2+} response to P2 signalling is seen during early retina formation and decreases in parallel with the decrease in mitotic activity. Incubation of cultured retina cells with known P2 receptor antagonists inhibits the uptake of labelled thymine, a base that constitutes part of the DNA molecule. In a similar manner to the process seen in astrocytes, P2 receptors in the retina are involved in the regulation of DNA synthesis (Sugioka *et al.*, 1999).

1.5.8. *Otocyst development*

The otocyst is the gravity detection system of the embryo. Studies of embryonic day three chick embryos have shown that Ca\textsuperscript{2+} ion fluxes can be
stimulated in the otocyst, caused by either acetylcholine or ATP application. This increase of intracellular Ca\(^{2+}\) ions may play a role in the differentiation of cells in the developing otocyst (Nakaoka and Yamashita, 1995).

1.5.9. *Branchial arch and somite development*

The chick P2Y\(_1\) gene was seen to be expressed in both branchial arches and somites during development. At stage 21, expression of the gene was seen in the mesenchyme of the mandibular process of both the 1st and 2nd branchial arches. The anterior expression was located in endoderm that lined the pharynx, whilst posterior expression was found on the ventral side of the mandibular process. Expression in the somites at stage 21 was seen in the anterior-most six somites. By stage 28, the expression spread to all but the most posterior somites, localised solely in the ventral myotome (Meyer *et al.*, 1999).

1.5.10. *Skeletal muscle development*

There have been a large number of studies conducted on the role of ATP and purinoceptors during skeletal muscle development. There have been several reports about P2 receptor expression in skeletal muscle (Ryten *et al.*, 2001, Meyer *et al.*, 1999, Wells *et al.*, 1995). A wide number of muscles in the developing chick (including the adductor, sartorius, gastrocnemius, biceps and triceps) have been shown to have a ubiquitous response to ATP during development, which is not seen in the adult muscle. The responsiveness was seen between stages 30 to 39 of development, but had disappeared by developmental stage 40. Either the purinoceptors expressed in the muscle have slowly declined in number, or the concentration of ATP is no longer large enough to drive the muscle fibres past the threshold (Wells *et al.*, 1995). The receptor subtype involved in this process has not been characterised. Thomas and Hume also found an ATP receptor expressed during chick skeletal muscle development (Thomas and Hume 1993). The extracellular ATP activates two classes of K\(^+\) ion channels which are important for correct skeletal muscle
1. General Introduction

development. The second messenger system used to couple the P2 receptors to K⁺ is thought to be a growth factor or a tyrosine kinase, as blockers to other G proteins did not inhibit the signalling. P2 receptor expression has been described in rat embryos and pups (Ryten et al., 2001). Using antibodies to detect the position of receptors, P2X₅, P2X₆ and P2X₂ were seen to be expressed in developmentally regulated patterns. The first appearance of these receptor subtypes mirrors the timing of key events in the development of skeletal muscle, including secondary myotube formation and maturation of neuromuscular junctions. It is possible that these P2X receptor subtypes have a role in controlling these developmental processes (Ryten et al., 2001).

1.5.11. Mesonephros development

The mesonephros is a group of tubules formed early in development that are the precursors of the kidney system. In fish and amphibians the mesonephros tubules develop into the renal tract. In mammals, the tubules degenerate and are replaced with a more complicated system. The chick P2Y₁ receptor was expressed in the mesonephros from stage 14 onwards. A clear cephalo-caudal gradient of expression was seen, which appeared to move down the body as development proceeded. By stage 25 it was only expressed in the tailbud. The expression appeared to reflect the maturity of the developing mesonephros. The P2Y₁ receptors were not seen to be expressed in the adult (Meyer et al., 1999).

1.5.12. P2 receptors in postnatal development

Most of the studies of P2 receptor signalling have been conducted on rat and mouse embryos (for reviews, see Burnstock, 2001, Hourani et al., 1999). The embryos are well characterised and good quality antibodies against P2 receptors has allowed the expression patterns and functions of these receptors to be well characterised. As this thesis looks at early expression patterns of P2 receptors during zebrafish development, the discussion of post-natal developmental roles will only be discussed briefly.
1.5.13. Brain expression during development

P2 receptors have been seen in a large number of brain areas during development (see Burnstock, 1999, Cheung and Burnstock, 2002). The expression appears to be developmentally regulated and varies with time. Specific areas that show expression include the paraventricular nucleus of the pituitary gland (Whitlock et al., 2001), the dorsal motor nucleus of the vagus and superior cervical ganglion (Ueno et al., 2001), the midbrain (where subtypes P2X1 to P2X6 were detected, Worthington et al., 1999). ATP has also been seen to be able to modulate the function of inspiratory neurons in the brainstem of neonatal rats (Funk et al., 1997). P2X3 receptors, one of the best studied subtypes of P2 receptor have been seen in a wide variety of brain areas. In the rat, for example, antibody staining against P2X3 protein was seen in the hindbrain, superior and inferior olives, mesencephalic trigeminal nucleus, intermediate reticular zone, spinal trigeminal tract and the hypoglossal nucleus (Kidd et al., 1998). Recordings from slices of rat neocortical brains in organ baths have also shown a role for ATP during brain development (Lalo et al., 1998). Application of ATP to rat neocortical neurons shows transient changes in the intracellular Ca^{2+} ion concentrations that vary with the age of the neurons. At postnatal day 13 (P13) a large response is seen to the ATP in pyramidal neurons of the sensorimotor cortex, which then decreases with time up to P30. The purinergic signalling system which is involved is thought to be elaborate and includes both P2X and P2Y receptors, although over 90% of the responses appear to be due the P2Y subtype. The transient changes in intracellular Ca^{2+} may be important for the transcription of genes that are needed for maturation of these pyramidal neurons. Several lines of research have shown that P2 receptors may have a role in the protection of hippocampal neurons from over stimulation by agonists. ATP has been shown to have two functions when added to cultured hippocampal neurons, inhibiting the release of an excitatory transmitter (glutamate) and stimulating γ-amino-butyric-acid (GABA, inhibitory) release. This is coupled to activation of potassium conductance through a G
protein, resulting in hyperpolarisation. Analysis of mRNA content from hippocampal neurons suggests that the signal is mediated by P2X receptors. ATP is thought to have a role in the prevention of excitotoxic cell death (apoptosis following over-stimulation) in hippocampal neurons (Inoue et al., 1998, Inoue, 1998). ATP and adenosine have also been shown to play a variety of roles in the metabolism of developing cells in the floorplate and roofplate. Both cell types act as a source of nucleotides that spread along the spinal cord during development (Yoshioka, 1989).

1.5.14. Heart and vasculature development

Whilst many studies have shown that adenosine acting on A1 receptors is important during the development of the heart and vasculature, the role of P2 receptors is not as well characterised (Bogdanov et al., 1998). A wide number of papers have shown expression of P2X\textsubscript{1}, P2X\textsubscript{3}, P2X\textsubscript{4}, as well as P2Y\textsubscript{2}, P2Y\textsubscript{4} and P2Y\textsubscript{6} in the heart, although the developmental function is not discussed. The major role of P2 receptors in the cardiovascular system is to function during platelet aggregation. This has already been discussed in the sections on P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors (Kunapuli et al., 1998). ATP has also been found to have a role in the control of the foetal heart. In the postnatal vascular system, both P2X and P2Y receptors are thought to be involved in the contractile response to sympathetic nerve stimulation (Phillips, 1998)

1.5.15. Lung development

The lungs of developing animals are filled with liquid that is secreted from the pulmonary epithelium into air spaces. The positive pressure that is formed is important for the expansion and development of the lung, the process being driven by Cl\textsuperscript{−} ion transport. Analysis of the expression patterns of P1, P2 and \(\beta\) adrenoceptors in foetal lung explants suggests that P2 receptors, amongst others, are involved in the control of modulating the secretion of Cl\textsuperscript{−} ions (Barker and Gatzy, 1998). Stimulation by ATP is seen early in rat development and is
thought to increase secretion from surfactant type II cells. Later on in development, both ATP and UTP can activate P2Y2 receptors in the lung to activate adenylate cyclase (Gobran and Rooney, 1997).

1.5.16. Gastrointestinal tract development

There have been several studies on the role of P2 receptors in gastrointestinal tract development (see Hourani, 1999, small intestine). ATP and ADP application to rat small intestine preparations causes different effects depending on the developmental age of the tissue. Early during development (postnatal day (PND) 1 to PND 7), application of ATP and ADP causes a contraction response. Later in development (PND 21 onwards) a relaxant response was seen to the same agonist application. This change in response to agonist is thought to mirror the changes that take place in the gut following weaning. The contractile response at this stage of development is provided by cholinergic transmission (Irie et al., 1994).

Similar contraction and relaxation responses to P2 agonists have been seen in other tissues of the gastrointestinal tract, including the rat colon (Hourani et al., 1993) and duodenum (Brownhill et al., 1997). The taenia coli has also been found to have developmental changes in P2 innervation (Zagorodnyuk et al., 1993). The expression of a zebrafish P2X4 receptor in the gastrointestinal tract will be described in chapter 4 of this thesis.

1.5.17. Vas Deferens development

The vas deferens develops later than the gastrointestinal tract and studies on P2 receptor expression in the vas deferens reflects this. As far back as 1970, the vas deferens was shown to produce excitatory postjunctional potentials in response to ATP (Furness et al., 1970). Responses to ATP in the rat vas deferens have been seen to develop from PND 15 onwards (Hourani et al., 1993). An increase in P2X1 receptor mRNA expression, for example, has also been demonstrated between PND 10 and PND 42 (Liang et al., 1998).
1.5.18. Development in other tissues

A few other tissues have also been found to have pharmacological profiles for P2 receptor activation during development, although no information is available about possible functions. These include the liver, urinary bladder and salivary glands.

1.6 Summary

Previous work has shown that there are two families of receptors that are activated by ATP: P2X ionotropic receptors and P2Y metabotropic receptors. Signalling through some P2X and P2Y subtypes has been seen to regulate several processes that may be important during embryological development, including apoptosis, cell differentiation, cell migration and cell proliferation.

In the first part of this thesis I describe the expression of three novel purinoceptors, P2X<sub>3</sub>, P2X<sub>4</sub> and P2Y<sub>11</sub> in the zebrafish, Danio rerio. The expression patterns of all three of the genes are described and discussed. Information is also presented that suggests that the P2X<sub>3</sub> receptor is involved in an early sensory response to ATP during development. This is consistent with its expression pattern at early developmental stages. The fish is a good model to look at the expression patterns of genes through different stages of development and also to consider evolutionary aspects of signalling in a family that has been studied in a wide variety of organisms.

The second part of the thesis describes the finding and characterisation of novel zebrafish mutants. Genetic screens in the zebrafish have been extremely successful, making it an important model for the study of vertebrate development. In the final part of this thesis I also describe the biological characterisation of monorail, a zebrafish midline mutant. The mutated gene is
1. General Introduction

mapped and sequenced to reveal the changes in the genetic code that underlie the phenotypic changes. This part of the thesis gives an overview of the process of generating, screening and characterising new mutants.
2. Materials and Methods

CHAPTER TWO

Materials and Methods

2.1. Fish maintenance and care

A colony of adult breeding zebrafish (*D. rerio*) were raised and kept as described in Westerfield (1993). The fish were kept on a 14 hour light / 10 hour dark cycle. Wild-type embryos were generated from the wild-type lines *uwt, *AB, Tübingen and Tup longfin. The recessive mutant lines *bonnie and clyde* (Kikuchi et al., 2000), *casanova* (Alexander et al., 1999), *faust* (Reiter et al., 2001), *monorail* (Brand et al., 1996), *smoothened* (Varga et al., 2001, Chen et al., 2001), *sonic-you* (Schauerte et al., 1998) *mzOEP* (Zhang et al., 1998) and *narrowminded* (Artinger et al., 1999) were also used. Mutant carriers of interest were identified by random intercrosses and were outcrossed to wild-type fish to maintain the line. Embryos were staged using morphological features (Kimmel et al., 1995).

Embryos were collected from either group matings of wild-type fish or heterozygous carriers of a mutation and allowed to develop at 28.5°C. Eggs were typically spawned at dawn and were collected and sorted before fixation. Developing embryos were kept in water supplemented with Methylene blue (Sigma). In order to generate non-pigmented embryos 0.2 mM phenylthioarbazamide was added to the water. Staged embryos were dechorionated and fixed using 4% paraformaldehyde (PFA, Sigma).

2.1.1. Observation of live embryos

Embryos were observed in fish system water under a Nikon dissecting microscope. Dechorionation was carried out manually using #5 gauge watchmaker's forceps. When required, embryos were anaesthetised with 0.02% tricaine (3-amino benzoic acidethylester) and immobilised for viewing in 3% methyl cellulose in fish system water.
2. Materials and Methods

2.1.2. Generation of transgenic fish and double mutant lines

Both transgenic lines and double mutant lines were formed by the crossing of two identified carrier fish. The monorail-Isllet1-GFP line was formed by crossing a heterozygous monorail carrier with a homozygous Isllet1-GFP fish (Higashijima et al. 2000). Therefore the generation which was produced was homozygous for the Isllet1-GFP and one quarter of the fish were heterozygous monorail carriers. The monorail and sonic-you double mutants were made by crossing a heterozygous monorail carrier with a heterozygous sonic-you fish (Schauerte et al., 1998). 1/16th of the new generation would be heterozygous for both monorail and sonic-you.

2.2. Molecular biology techniques

Standard molecular biology techniques were carried out according to Sambrook et al., (1989). For large scale DNA preparations, plasmids were transformed into E. coli (JM109 strain) competent cells and cultured in 100 ml LB medium supplemented with ampicillin (Sigma; 80 μg/ml). DNA was purified using a Qiagen midiprep column kit (Qiagen).

Restriction enzyme digests were carried out accordingly in 20-100 μl volumes using an appropriate 1x enzyme buffer and 2-5 units of enzyme per 1 μg of template DNA. Digests were incubated at 37°C and checked by electrophoresis on a 1% agarose TAE gel (Sigma). Digests were cleaned when necessary. Purification of DNA was carried out by 2x phenol:chloroform extraction and 1x chloroform extraction followed by precipitation. DNA was precipitated with 0.1 volume 3M NaAC and 2.5 volumes 100% ethanol at -20°C, for 30 minutes. Precipitated DNA was centrifuged for 15 minutes and the resulting pellet was washed in 70% ethanol, air dried and resuspended in either TE buffer (10 mM Tris-HCL, pH 8.0 and 1 mM EDTA) or ultrapure water where appropriate. Solutions were stored at -20°C until needed, to prevent degradation of the template.
2. Materials and Methods

2.2.1. Novel P2 receptor genes

Novel P2 receptor genes were obtained from the Washington University expressed sequence tag (EST) database (www.wuzgr.edu). The database was searched using a basic length sequence alignment (BLAST) programme which identified novel P2 genes by their homology to paralogues in other species. Genes obtained from the EST database were kept as glycerol stocks. On arrival in the lab, the stocks were streaked onto LB agar (Sigma) plates containing antibiotic (typically ampicillin, 80 µg/ml: Sigma) and were then prepared by a midiprep. The clones were sequenced to confirm their identity. In some cases the genes had to be subcloned into different plasmids to allow the easy transcription of RNA from promoters. EST clones were digested and then blunt end ligated into a pBluescript SK+ plasmid.

2.2.2. Bacterial stocks

Long term bacterial stocks were made by addition of bacterial containing LB broth (Sigma) to autoclaved glycerol (Sigma). One volume of bacterial colony was added to one volume glycerol and the solutions were mixed. Glycerol stocks were stored at -80°C. Small amounts of the glycerol were plated out onto an LB agar plate to grow new colonies containing the gene of interest when needed.

2.2.3. Primer design

Specific primers were designed using the Gene Jockey II sequence analyser programme (Biosoft). Sequences were chosen that would give primers with optimal melting temperatures when used in a polymerase chain reaction (PCR). Primers tended to be around 21 base pairs long to provide a suitable annealing temperature of around 55°C. Primers were synthesised by GibCo BRL, and were stored at -20°C in ultrapure water at a stock concentration of 100mM.
2. Materials and Methods

2.2.4. Polymerase chain reaction

Genes were amplified using the polymerase chain reaction (PCR). A typical PCR mixture had a total volume of 20μl and contained 14.2μl ultrapure water, 2μl buffer (with 2.5 μm magnesium included), 1μl forward and reverse primer (concentration 20μm), 0.5μl dNTPs, 0.3μl Taq polymerase (Promega) and 1μl of template. A typical PCR reaction would have 25 to 30 amplification cycles (94°C for 1 minute; annealing temperature 1 minute; 72°C, 1 minute). The annealing temperature and length of extension time were titred for each reaction. The resulting products were visualised on a 1% TAE agarose gel containing ethidium bromide and were then cloned into the pCRII-TOPO vector using a TA cloning kit (Invitrogen). Specific PCR reaction conditions will be given in appendix 1 for each experimental condition.

2.2.5. Reverse transcription polymerase chain reaction

RNA for reverse transcription polymerase chain reaction (rtPCR) was prepared using a Qiagen RNeasy kit (Qiagen). The yolk sac was removed from pools of 100 embryos at 24 hours of development and the embryos were snap frozen in liquid nitrogen and stored at -80°C. The embryos were homogenised using a QiaShredder column (Qiagen) and the homogenate was used in an RNeasy spin column reaction. cDNA for reverse transcription was manufactured using a reverse transcription kit (GibCoBRL). The cDNA was used as a template in subsequent PCR reactions. In some cases, the cDNA that was made incorporated 2pmol/L of a gene specific primer instead of being oligo dT primed. This increased the likelihood of the primers binding to the template. PCR reactions were performed in a similar way to the protocol described above.

2.2.6. Sequencing

DNA was sequenced at the Advanced Biotechnology Centre, Imperial College London. The returned sequences were analysed using the Gene Jockey II sequence processor (Biosoft) and compared to other known sequences using the BLAST program on the genbank database to determine
2. Materials and Methods

their identity. The primer sequences were removed from the end of the insert and the remaining sequence was then analysed.

2.2.7. Tree diagrams

Tree diagrams of known P2X and P2Y receptor genes from a variety of species were made using Tree Puzzle software obtained from the internet. A viewing program (tree-view) was also used to produce a neat tree (Stimmer and von Haeseler, 1996). The chosen sequences were aligned using a clustal W alignment in Gene Jockey II (Biosoft). Areas of low homology were removed and a tree was produced according to the quartet maximum likelihood method.

2.3. Embryological techniques: Detection of gene and protein expression

2.3.1. Preparation of DNA templates

DNA templates were linearised for in situ hybridisation probe transcription. The appropriate template was digested with a restriction enzyme to produce a linearised piece of DNA. The digestion was terminated by cleaning with a phenol:chloroform mixture followed by precipitation. The pellet was resuspended in between 10 and 20μl ultrapure water and 1μl of the template was run on a 1% agarose TAE gel containing ethidium bromide to assess the concentration of the digested DNA.

2.3.2. Transcription of RNA probes

In situ hybridisation probes were made by in vitro transcription from the digested DNA template. 1μg of linear DNA was used in the in vitro transcription reaction to synthesise antisense RNA labelled with digoxygenin-2-UTP. A 10μl reaction was set up containing transcription buffer (200mM Tris-HCL pH7.5, 30mM MgCl2, 10mM spermidine, 50mM NaCl); 10mM DTT; NTP-digoxygenin mix (1mM ATP, CTP, GTP, 0.65mM UTP, 0.35mM UTP-digoxygenin, Boehringer Mannheim); 40 units RNasin (Promega); 10 units T3/T7/SP6 RNA polymerase. The transcription mix was incubated at 37°C for 2 -4 hours. RNA
2. Materials and Methods

was then precipitated by addition of 900μl 100% ethanol (Sigma) and 100μl 7.8M ammonium acetate and 190μl ultrapure water, -20°C for 1 hour. The precipitated RNA was centrifuged 14,000, 15 minutes. After washing with 70% ethanol, the RNA was centrifuged again for a further 15 minutes, 14,000 rpm. The resulting pellet was vacuum dried and then resuspended in 200μl ultrapure water. The suspension was spun through a Millipore "iilac" column (Ultrafree MC, Millipore) which concentrated RNA above the column and allowed unincorporated nucleotides and excess water to pass through. The RNA was washed with 200μl ultrapure water, leaving the RNA in a 20μl volume above the column. The probe was stored at -20°C and diluted 1:200 in hyb+ mixture (see below for recipe) before use.

2.3.3. Wholemount In situ hybridisation

Embryos used for in situ hybridisations were staged and dechorionated prior to fixation in 4% paraformaldehyde (PFA, Sigma). Fixation was overnight, 4°C. The fixed embryos were washed twice in PBSTx (PBS tablets, Oxoid, containing 0.05% Triton X100 (BDH)) and then placed in 100% methanol at -20°C for one hour or more to prevent RNA degradation. Embryos were left in methanol for periods of up to one month.

On the first day of the protocol embryos were rehydrated by washing with a mixture of 50% methanol, 50% PBSTx for 5 minutes, and were then washed 4 times in PBSTx, 5 minutes per wash. To permeabilise embryos, a solution of proteinase K (0.01 mg/ml, Sigma) was used for an appropriate period of time according to the stage of embryo. Typically, tailbud stage embryos were digested for one minute, 24 hour embryos for 10 minutes and 48 hour embryos for 20 minutes. The digestion time was altered according to the strength of proteinase K. Embryos were postfixed in 4% PFA for 20 minutes and were then washed another 5 times in PBSTx, 5 minutes per wash. Samples were incubated in hyb+ (50% formamide, 5X SSC, 500μg/ml yeast RNA, 50μg/ml heparin and 0.1% Tween-20 brought up to pH 6.0 with 1M citric acid) for an hour.
or more at 68°C. Following prehybridisation, the hyb+ was replaced with \textit{in situ} probe diluted in hyb+ and the embryos were left overnight at 68°C. A typical probe dilution was 1:200, dictated by the time needed for \textit{in situ} hybridisations to develop.

On the second day of the protocol, the probe was removed and stored at –20°C to be used a second time. The probe was replaced with a hyb- solution (25ml formamide, 12.5ml 20X SSC, 500μl 10% Triton X100, 12.45ml autoclaved water) and incubated for a further 2 hours, 68°C with several changes of hyb-.

The embryos were washed with two quick changes of 2X SSC and then were washed for one hour in 0.2X SSC at 68°C. The next step was to wash in PBSTx for one hour at room temperature with several changes. The samples were blocked for one hour or more in maleic acid block (One litre maleic acid buffer contains 11.61g maleic acid, 8.77g NaCl, brought up to pH 7.5 with NaOH. The maleic acid block was made by addition of 2% Boehringer blocking powder).

The MABI was replaced with anti dioxygenin-2-UTP FAB fragments antibody (Roche Diagnostics) diluted 1:6000 in MABI. The embryos were incubated overnight in antibody at 4°C.

On the third day the antibody solution was removed and replaced with PBSTx. The embryos were washed with many changes of PBSTx over a period of 2 hours at room temperature. The final step was to wash the embryos in developing solution (0.292g NaCl, 0.49g MgCl, 5ml 1M Tris/Hcl pH 9.5, 45ml ultrapure water) for 3 washes, 5 minutes at a time. The \textit{in situ}s were left to develop in 500μl BMPurple solution (Boehringer Mannheim) in the dark until staining was complete. The embryos were postfixed for 20 minutes in 4% PFA and then washed into 70% glycerol for storage and photography.

\textbf{2.3.4. Wholemount double \textit{in situ} hybridisation}

Double \textit{in situ} hybridisation was performed by the addition of two probes at the hybridisation stage. The first probe incorporated digoxigenin-2-UTP.
2. Materials and Methods

labelling mix (Boehringer Mannheim) and the second fluoroscein-2-UTP
labelling mix (Boehringer Mannheim) during the in vitro transcription reaction.
The two antibody reactions to detect RNA transcription were performed
sequentially, with a short fixation step (a pH 2 glycine buffer rinse) in between
them. The anti-fluoroscein alkaline phosphatase (Boehringer Mannheim) was
used first, followed by anti-digoxigenin alkaline phosphatase (Boehringer
Mannheim). Developing was also sequential, using first BCIP IMP (Boehringer
Mannheim) to give a red stain and then BMPurple (Boehringer Mannheim) to
stain in blue. Embryos were post fixed and photographed.

2.3.5. Plastic sections of whole mount in situ hybridisations.

Embryos were washed out of storage in 70% glycerol by three 10 minute
washes with water. Embryos were then washed into methanol using methanol /
water solutions for 2 minutes at a time: 2 washes each of 30% methanol; 50%
methanol; 70% methanol; 95% methanol; 100% methanol. Solution A (Agar
scientific) was activated by the addition of 0.225g benzoyl peroxide to 25ml
solution A (subsequently called activated solution A). The embryos to be
sectioned were embedded according to the protocol provided with the JB4
sectioning kit (Agar scientific). A microtome was used to cut 10μm sections of
these blocks and the sections were dehydrated onto compartmented glass
slides (Hendley LTD) on a heat block. The dried sections were mounted using
DPX mounting medium (BDH) and were allowed to dry for 48 hours before being
photographed on a compound microscope (Nikon)

2.3.6. In situ hybridisation on cryostat sections

Embryos for cryo-sectioning were rinsed in phosphate buffer (PBS,
Oxoid) and equilibrated in 10% and 20% sucrose in PBS at 4°C over 48 hrs.
Tissue was embedded in OCT compound (Agar scientific), frozen on dry ice and
cut serially at 20 μm in coronal and sagittal planes. Sections were collected on
Superfrost slides (BDH) and stored at −70°C in sealed boxes until ready to use.
2. Materials and Methods

The *in situ* hybridisation procedure on cryostat sections was carried out as follows. Sections were air-dried at room temperature for 20 minutes to 3 hours and postfixed with 4% paraformaldehyde in phosphate buffer containing 0.1 M NaCl (PBS) for 20 minutes. Following 3 washes in PBS, sections were acetylated and incubated in 50% formamide, 3X SSC until ready to be exposed to the riboprobe. Riboprobes were diluted to 100 ng/ml in warm (60°C) hybridisation solution, containing 50% formamide, 5X SSC, 10 mM beta-mercaptoethanol, 10% dextran sulphate, 2X denhardt's solution, 250 µg/ml yeast tRNA, 500 µg/ml heat inactivated salmon sperm DNA. Hybridisation was carried out in a humid chamber at 58°C for 16 hours. Slides were rinsed in 50% formamide, 2X SSC at 58°C, treated with RNAse A and RNAse T1 at room temperature, rinsed twice with 50% formamide, 2X SSC at 58°C and incubated with anti-digoxigenin antibody as above. Following development of the colour reaction slides were dehydrated and mounted in DPX (BDH).

2.3.7. cDNAs used for in situ hybridisation


2.4. Immunohistochemistry on wholemount embryos

2.4.1. Antibody staining

Embryos for antibody stains were fixed in a 2% trichloroacetic acid (TCA, Sigma) solution for three hours exactly. After fixation, the embryos were washed several times in PBS before storage at 4°C. Some antibody protocols
used 4% paraformaldehyde as the fixative, primarily on embryos that were younger than 48 hours.

Immunohistological methods were based on those developed by Wilson et al., (1990) and MacDonald et al., (1995).

Embryos were rinsed several times in PBSTx (phosphate buffer saline (Oxoid) with 0.8% Triton-X100 (BDH)) at room temperature to remove the fixative. Embryos older than 48 hours were permeabilised by incubation in a 2.5 mg/ml solution of trypsin for 4 minutes on ice. This was followed by extra washes in PBSTx. Embryos were incubated in 10% foetal calf serum or normal goat serum for one hour or more at room temperature in order to block the non-specific binding of the antibody. The 10% serum was then replaced with a primary antibody solution diluted in 1% normal goat serum and were left overnight at 4°C. The following day, the embryos were washed over several hours with frequent changes of PBSTx and were then incubated in the secondary antibody (Sigma) diluted in 1% foetal calf serum or normal goat serum. Generally, secondary antibodies were used at a dilution of 1:200. The embryos were left overnight at 4°C in secondary antibody solution. On the third day, the embryos were again washed several times in PBSTx over a number of hours before processing and visualisation of the bound antibodies. For horseradish-peroxidase (HRP) conjugated antibodies, the embryos were incubated in 2mM dianinobenzadine (DAB; Sigma) in PBS for 10 minutes at room temperature prior to the addition of 1-2 μl of 3% H₂O₂ (Sigma). Once the reaction had been seen to have gone to completion, as seen by the appearance of brown reaction product, the reaction was terminated by PBS washes and the embryos were post-fixed in 4% paraformaldehyde for 2 hours or more at room temperature. Embryos were then cleared through a series of glycerol washes and stored in 70% glycerol for observation, dissection and microscopy.
2. Materials and Methods

Primary antibodies used in this thesis include anti-acetylated tubulin (Wilson et al., 1990); anti-HNK1 (Wilson et al., 1990); anti-opsin (Stenkamp et al., 1998); anti-Islet1 (Korzh and Thor, 1993) and anti-GFP (AMS Biotechnology), and anti-ZRF1 (Hughes et al., 1995). Secondary antibodies were anti-mouse IgG for all reactions except for HNK1, for which anti-mouse IgM was used and anti-GFP for which anti-rabbit IgG was used.

2.4.2. Backfills of hindbrain reticulospinal neurons

Backfills of reticulospinal neurons were performed by the application of lysinated rhodamine dextran (LRD, from Molecular Probes) to the spinal cord. Embryos were dechorionated and left to develop for four or five days at 28°C. Before backfilling, embryos were anaesthetised in a solution of MS222 (Tricaine, Sigma) and were placed on a sylgard dissection plate. The embryos were placed inside a small drop of liquid which held them in position by surface tension. A sharpened tungsten dissection pin was used to lesion the spinal cord at the level of the end of the yolk extension. LRD was applied to the wound site and the embryos were placed in a fresh dish of fish water to recover. After a delay of 30 minutes in order to enable the dye to track up the spinal cord the embryos were fixed overnight in 4% PFA, 4°C. The following day, the brain was removed from the fixed embryos and mounted ventrally to allow imaging with a confocal microscope.

2.5. ENU mutagenesis screen protocols

2.5.1. Generation of founder fish - ENU mutagenesis

The mutagenesis method used in both the UCL screen and the Tübingen 2000 screen was based on Mullins et al., (1994) and Haffter et al., (1996). Adult male fish were treated with ENU (ethylnitrosourea) in order to induce point mutations in the sperm nucleus DNA. Males were paired with females the day before mutagenesis and only those that successfully fertilised eggs were selected for mutagenesis treatment. A stock solution of ENU (Sigma) was dissolved in 10mM acetic acid to a concentration of 100mM. For use, the ENU
was further diluted into a 10mM sodium phosphate buffer (pH 6.0) to make a final concentration of 3mM. Fish were placed into a mouse cage containing the ENU solution for three periods that lasted one hour. The procedure was repeated every day for one week. Following each treatment, the fish were washed by passing them through two tanks of clean water and were then left to rest and recover. Two weeks after completion of the treatment, the fish were paired with untreated wild-type females and the resulting eggs were discarded. This removed any sperm that were in the spermatogonia during the mutagenesis regime and so would carry mosaic copies of mutated genes. Following this the males were ready to be used to generate an F1 generation of fish.

2.5.2. Mutant crossing scheme

Mutagenised male fish were crossed to wild-type females, resulting in F1 fish that all carried one mutation per genome. These fish were then inbred to produce an F2 generation that had a total of two mutagenised genomes in their background. 50% of the fish were heterozygous for the induced mutation. F2 fish were mated randomly to produce a screenable F3 generation. On average, in one quarter of the matings both parents were carriers of the mutation, and therefore their progeny would be one quarter homozygous mutant F3 embryos. If the mutated gene affected a process that was developmentally interesting, the F2 generation parent fish were kept and repaired to enable heterozygous carriers to be identified (Fig. 2.1).

2.5.3. Screening protocol, UCL screen

Founder fish were generated using a standard regime of ENU treatment and were allowed to recover before being used to generate F3 generation embryos for screening. Embryos were screened at 3 somite stage, 24 hours, 48 hours and 72 hours to detect morphological defects. Embryos were dechorionated by hand and were then fixed at 48 hours in 2% trichloroacetic acid (TCA). A standard tubulin and opsin antibody staining protocol was used (see
Figure 2.1. Breeding scheme to show the production of mutagenised zebrafish embryos for screening. The protocol for treating the male founder fish is described in the text. F3 generation embryos that are clear represent wild-type fish and those coloured in brown represent mutant fish. The scheme is modified from Hafter et al., Development vol. 123, 1996.
2. Materials and Methods

Material and Methods). Embryos were screened for morphology defects and staining pattern defects using Nikon dissection microscopes.

2.5.4. Screening protocol, Tübingen screen 2000

Adult F2 generation fish from ENU treated founder males were paired with siblings on Wednesday afternoon. Embryos were collected on Thursday morning. A minimum of 20 embryos per clutch were required for screening. The embryos were grown up at 31°C overnight, with addition of 0.2mM phenulthiourea (PTU) to the water at 10pm, following completion of gastrulation.

On Friday morning, all clutches were screened for morphological defects (at around 24 to 30 hours of development) using dissection microscopes (Leica or Zeiss). Dead embryos were removed and recorded, and pronase (0.04mg/ml) was added to each dish. Embryos were left at room temperature for 3 to 6hrs. When all embryos were out of their chorions, and had reached 32hrs of development (staged by morphology), they were placed in 4% paraformaldehyde (PFA) and fixed overnight at 4°C.

On Saturday morning, embryos were washed into PBTx (phosphate buffered saline with the addition of 1%Triton X-100 (BDH)) for 2 hours, changing the washing solution every 10 to 15 minutes. Embryos were then blocked in PBTx with 10% goat serum (Sigma) for 4 hours at room temperature on a shaker and then incubated in primary antibody solution (PBTx with 1% goat serum and anti-tubulin antibody (Sigma) at a concentration of 1:1000 / anti-opsin antibody at a concentration of 1:500) overnight at 4°C on a mechanical shaker.

The following morning, the primary antibody solution was removed and embryos were washed in PBTx for 2 hours, with changes of solution every 10 to 15 minutes. Embryos were incubated in secondary antibody solution (PBTx with 1% goat serum and anti-mouse IgG (Sigma) at a concentration of 1:200) for 4 hours at room temperature on a shaker. Embryos were washed in PBTx for 2
2. Materials and Methods

hours with a change of solution every 10-15 minutes. The embryos were then
developed using Sigma Fast 3,3’-Diaminobenzidine tablet sets (1 tablet of H₂O₂
and urea and one tablet of DAB in 15ml PBS). Following development, embryos
were washed 5 times for 5 minutes in PBTw (PBS with 0.1% Tween-20, Sigma),
then 5 minutes in PBS, then in 50% glycerol (50% PBS) and finally into 70%
glycerol in which the embryos were screened and stored.

2.5.5. Analysis of fluorescent antibody staining and GFP expression

Fluorescent signals in both live and fixed embryos were assayed by
examination under a fluorescent dissecting microscope (Leica). Embryos were
embedded upside down in 2% low melting point agar and were then inverted to
allow photography of the plane of interest. This minimised the amount of agar
between the embryo and the microscope lens. Pictures were taken using Kodak
slide film in a Kodak camera.

2.6. Preparation of morpholinos and RNA for injection

2.6.1. Cloning of foxA1 into PCS2+

For synthesis of RNA for injection, foxA1 and twhh were cloned into
PCS2+, a derivative of pBluescript. Both foxA1 and twhh were excised from
pBluescript SK+ by a double restriction digest using BamH1 and Xho1. PCS2+
was digested using the same enzyme mixture and both digests were gel purified
and band prepared using a gel extraction column (Millipore Ultrafree DA
column). The concentration of prepared insert and plasmid were determined by
electrophoresis on a 1% agarose gel containing ethidium bromide. Comparison
to a 1Kb DNA ladder of a known concentration (from Promega) allowed the
concentration of DNA to be calculated.

2.6.2. Ligation of prepared templates into PCS2+

The ligation reaction was typically carried out in a 20μl reaction volume.
40ng/μl of vector and 40ng/μl of insert were added to 4μl of buffer and 1μl of T4
DNA ligase. Ultrapure water was added to make a final volume of 20μl. For

94
example, 5µl of foxA1 template at a concentration of 10ng/µl were added to 4µl of PCS2+ at a concentration of 12.5ng/µl along with 4µl buffer, 1µl enzyme and 6µl water. All ligation reactions were incubated in a water bath at 16°C for around 24 hours. The next evening, the ligated template was immediately transformed into competent cells. 2µl of the ligation reaction were gently mixed with a vial of JM109 cells (Sigma) and were left on ice for 5 minutes. The cells were heatshocked in a 37°C water bath for 90 second and were then quenched on ice. 500µl of LB broth (Sigma) was added to the cells and the transformation was allowed to recover in an automatic shaker at 37°C for 30 minutes. The cells were finally plated out onto LB agar plates (Sigma) containing antibiotic and were grown overnight at 37°C. The next morning, colonies were picked using a sterile pipette tip and the colonies were grown up in LB broth for 12 hours at 37°C in a mechanical shaker. The DNA was finally purified using a Qiagen spin miniprep kit (Qiagen).

2.6.3. In vitro transcription of mRNA for injection

*In vitro* transcriptions were carried out using the Ambion Megascript Kit (Ambion). In a total reaction volume of 20 µl, 1 µg of linear DNA template was added to 1x transcription buffer, 5mM ATP, 5mM CTP, 5mM UTP, 1.5mM GTP, 6mM 7mG(5') ppp(5')G sodium salt (capGTP), and 2 µl SP6 or T3 RNA polymerase enzyme mix. Transcription reactions were incubated for 2 hours at 37°C. The DNA template was removed by a 15 minute incubation with DNase.

The transcription reaction was terminated by the addition of 190 µl dH2O, 900µl 100% ethanol and 100µl 7.8M NH4AC. The precipitation was incubated at -20°C for one hour and then spun in a chilled centrifuge at 14,000 rpm, 4°C for 20 minutes. The resulting pellet was washed with 1000µl 70% ethanol and was spun for a further 15 minutes, 14,000 rpm at 4°C. The pellet was vacuum dried and resuspended in 200µl ultrapure water, prior to being cleaned in a Millipore Ultrafree MC column (Millipore). The concentration of RNA was measured in a
2. Materials and Methods

spectrophotometer (Ambion) and was diluted in ultrapure water before injection. Typically the concentration of RNA that was injected was around 20ng/µl.

2.6.4. Preparation of morpholinos for injection

Morpholinos were synthesised on request by Gene-tools inc. Typically, a morpholino was designed against the first twenty base pairs of the coding region of the gene immediately following the initiation methionine. Morpholinos were diluted to a stock concentration of 2 mM in Danieau solution (58mM NaCl, 0.7mM KCl, 0.4mM MgSO₄, 0.6mM Ca(NO₃)₂, 5.0 mM HEPES pH 7.6). The stock concentration was stored at -20°C until use. For injections, the concentration of injected morpholino was titred to allow phenocopying of the mutant without causing side effects such as necrosis and general body shape defects. Typically, working concentrations of morpholinos ranged from 0.1mM to 1mM. Morpholinos against p2x3, twhh, shh and foxA1 were used in this thesis.

2.6.5. Injection of morpholinos and RNA

Embryos that had been left in their chorions were aligned in a trough formed by the edge of a glass slide (BDH) in the inverted top of a 50ml petri dish (Falcon). RNA or morpholino was injected in a volume of approximately 200 pl into one cell of a 1-4 cell stage embryo, using a glass capillary needle attached to a gas driven microinjector (Nashirige Co. Japan). The injected embryos were left to develop in fish water at 28.5°C and unfertilised embryos were discarded at the blastula stage. The number of remaining embryos were recorded.

The amount of foxA1 RNA injected was generally between 10-100 ng/µl. Morpholinos against p2x3 and foxA1 were injected at a concentration of 1mM.

2.7. Mapping of genes

Mapping of the monorail mutant was done by Maryam Mangoli in Michele Rees's group, Rayne Institute, UCL. Mapping was performed using a simple
2. Materials and Methods

sequence length polymorphism (SSLP, from ResGen) kit and Taq DNA polymerase (Sigma). Following identification of the candidate gene underlying the monorail mutant, primers were designed to amplify the whole foxA1 open reading frame. The primers were synthesised by GibcoBRL. foxA1 was amplified from single wild-type and mutant embryos and the fragment obtained was cloned into the Topo10F' (Invitrogen) before being sequenced. The mapping strategy is discussed in more detail in chapter 7 of this thesis.

2.8. Photography and image analysis

All photographs were taken using either a dissection microscope (Nikon) or compound microscope (Nikon) coupled to a digital camera (Polaroid). Embryos were dissected to remove the yolk and any necessary brain structures and were then flat mounted in 100% glycerol between a slide and a coverslip. Images were processed and arranged using Adobe Photoshop version 5.5 (Adobe).
3. Embryonic expression of a P2X<sub>3</sub> receptor in zebrafish

CHAPTER THREE

Embryonic expression of a P2X<sub>3</sub> receptor in zebrafish

3.1. Abstract

From studies performed primarily in mammals, it is thought that the P2X<sub>3</sub> purinoceptor is involved in mediating sensory and nociceptive signals in adult tissues. However, little is known concerning the expression or function of P2X family genes during early development. This chapter describes the expression of a gene (p2x3) encoding a P2X<sub>3</sub> receptor during zebrafish development. It is reported that zebrafish P2X<sub>3</sub> is expressed in the anlage of the trigeminal ganglion from very early stages of development, most likely in neural crest derived trigeminal cells as opposed to placode derived trigeminal cells. p2x3 is also expressed in the spinal sensory Rohon-Beard cells and in the putative posterior lateral line ganglion.

3.2. Introduction

P2X<sub>3</sub> receptors have been cloned from a wide variety of species and both the pharmacology and tissue distribution are well documented (Boué-Grabot et al., 2000, Egan et al., 2000). Analysis of the P2X<sub>3</sub> knockout mice has also helped elucidate the function of this receptor (Cockayne et al., 2000). In this chapter I present the expression of a novel zebrafish P2X<sub>3</sub> homologue and an attempt is made to assess the function of the zebrafish receptor. A more complete description of the pharmacology and function was included as part of the general introduction in chapter 1.

3.3. Pharmacology

The pharmacology of P2X receptors shows wide variation between different species, making it impossible to predict agonist binding efficiencies and antagonist potencies for new cloned receptors. The zebrafish P2X<sub>3</sub> receptor
3. Embryonic expression of a P2X3 receptor in zebrafish

has been independently cloned by another group and has been shown to have an agonist profile that is closest to that of P2X7 receptors, with the largest response being seen after activation by benzoylbenzoyl ATP (bzATP, Boué-Grabot et al., 2000). The channel shows the fastest activation time and kinetics of desensitisation of any P2X receptor studied so far. Generally, zebrafish P2X3 is highly sensitive to block by antagonists such as TNP-ATP, whereas suramin only has weak activity (Boué-Grabot et al., 2000).

The responses of agonists and antagonists applied to the zebrafish P2X3 receptor place it in a group of P2X receptors that show fast desensitisation, high sensitivity to ATP and α,β meATP and strong inhibition by TNP-ATP (Virginio et al., 1998). The zebrafish P2X3 receptor, in a similar way to P2X3 receptors in other species, is known to be able to form heteromultimeric channels with the P2X2 receptor. This was tested by coexpressing zebrafish P2X3 with rat P2X2 (the zebrafish P2X2 receptor is not yet available) and measuring the pharmacological properties (Egan et al., 2000). The responses measured after α,β me ATP application were as would be expected from a heteromultimeric receptor: the response was smaller than that of a P2X3 homomultimer and greater than the response recorded from a P2X2 homomultimer (Egan et al., 2000).

3.4. Tissue distribution of the P2X3 receptor

Cell bodies of sensory neurons, including the trigeminal, nodose and dorsal root gangliaions show P2X3 expression in a subset of small diameter crest-derived sensory neurones (Chen et al., 1995, Tsuda et al., 1999). There have been many studies implicating P2X3 receptor in the transduction of pain (Burnstock, 2001, Tsuda et al., 2000, Cockayne et al., 2000). P2X3 receptor expression has also been reported in taste buds and cochlear and vestibular ganglia of rats (Bo et al., 1999, Xiang et al., 1999). The expression in sensory ganglia co-localises with both the vanilloid receptor 1 (VR1, activated by
3. Embryonic expression of a P2X₃ receptor in zebrafish

capsaicin), calcitonin gene related peptide (CGRP) and Isolectin IB4 (Bradbury et al., 1998, Guo et al., 1999, Tsuda et al., 1999).

The enteric nervous system has also been shown to have high levels of P2X₃ receptor expression. Antibodies against P2X₃ receptors in rat and mouse have shown expression on lumbrosacral afferents (pelvic afferents) of the bladder. P2X₃ receptors are found to be localised to the smaller, myelinated Aδ fibres of pelvic afferents, suggesting the receptor has a role in the bladder emptying reflex loop (Yiangou et al., 2001).

The study of the function of the P2X₃ receptor has been helped by the generation of knock-out mice and studies on changed expression and function of the receptor during several disease states (Cockayne et al., 2001, Vlaskovska et al., 2000, Moore et al., 2001, Yiangou et al., 2001b). Knock-out mice show bladder hyporeflexia, which is characterised by increased bladder voiding time and an increased retention of urine in the bladder. In models of inflammatory disease such as inflammatory bowel disease (IBD) or idiopathic detrusor instability (IDI), the receptor expression levels are either increased (IBD) or decreased (IDI). These studies were described as part of the general introduction in chapter 1.

3.5. Results
3.5.1. Molecular characteristics

An EST clone that showed high homology to P2X₃ receptors of other species when compared using a BLAST (Basic Local Alignment Search Tool) was obtained from the Washington University zebrafish EST project. The genbank accession number for the EST clone is AI588766. The p2x3 gene has open reading frame that is 1724 base pairs long and codes for a protein of 416 amino acids. The zebrafish receptor shares only 54% homology to the closest related mammalian homologue (Fig.3.1.). A similarity tree based on the amino acid sequences of various P2X genes confirms that the zebrafish gene encodes
Figure 3.1.

Tree diagram showing the relationship between the zebrafish P2X3 receptor and receptors from various other species. The closest related receptors to the zebrafish gene are the human and rat P2X3. The accession numbers are: P2X3 zebrafish, AF239292; P2X3 human, AB016608; P2X3 rat, AF084975; P2X2 rat, AF020756; P2X2 human, AF190822; P2X2 guinea pig, AF05332; P2X4 rat, U47031; P2X4 mouse, AF089752; P2X4 zebrafish, AF317643; P2X4 human, XM_045292; P2X4 chick, AF218449; P2X4 frog, AF308148; P2X6 human, AF065385; P2X5 human, AF016709.
3. Embryonic expression of a P2X₃ receptor in zebrafish

for a purinoceptor. The highest homology was seen to both the human and rat P2X₃ receptors (Fig 3.1.).

3.5.2. In situ hybridisation expression pattern

Expression of the zebrafish p2x3 gene starts around the 6 somite stage in two lateral stripes adjacent to the dorsal neural keel and caudal to the eye (Fig. 3.2.A,B). This position is consistent with the expressing cells being cranial neural crest. A pair of bilateral spots of stronger expression at the posterior end of the stripes (between the developing eye and ear) persists throughout development (Fig. 3.2C,D). At around the 14 somite stage, weak and transient expression is visible in the developing cells of the rostral diencephalon and telencephalon (Fig 3.2F) and in scattered cells within the spinal cord (Fig 3.2H). Both sites of expression disappear shortly after this stage, although it is possible that either all, or a subset of the cells labelled in the trunk are Rohon-Beard cells.

During late somitogenesis, expression in sensory Rohon-Beard neurons in the spinal cord becomes apparent (Fig 3.2I). Rohon-Beard neurons are easily recognisable due to their prominent size and position in the dorsal neural tube (Grunwald et al., 1988, Metcalfe et al., 1990). p2x3 expression at this site decreases over time consistent with apoptotic depletion of Rohon-Beard neurons during the second day of development (see William et al., 2000). A double staining with Islet-1 antibody, which is known to label both Rohon-Beard neurons and primary motorneurons confirms this identity (Fig. 3.3.).

The domain of p2x3 expression between the eyes and ears elongates slightly at 20 hours post-fertilisation (hpf), frequently fragments into two groups of cells around 24 hpf (Fig. 3.2I) and coalesces again to a single domain by around 30 hpf (Fig. 3.3A). By 50 hpf, the rostral domains of expression are adjacent to the eye and a second pair of bilateral expression domains appears at the anterior border of the first somite, just caudal to the ear. This is the
3. Embryonic expression of a $P2X_3$ receptor in zebrafish

Figure 3.2. legend

Early early expression of p2x3 in putative central and peripheral neural cells. Lateral (A,C,F-I) and dorsal (B,D,E, with anterior down) views of embryos showing p2x3 expression at the stages indicated. (A,B) Expression in dorsal cells adjacent to the midbrain and anterior hindbrain. The arrowheads indicate spots of higher expression. (C-H) Twelve somite stage embryos showing expression in putative trigeminal ganglion cells (arrowheads, C,D,F) in the forebrain (G) and in various spinal cord cells (E,H). (I) Twenty-four hour embryo in which the expression in the putative trigeminal ganglia cells has condensed to two spots (arrows) and in which expression in dorsal Rohon-Beard neurons is prominent (the inset shows a dorsal view of the spinal cord). Abbreviations: e, eye; d, ventral diencephalon; o, otic vesicle; t, telencephalon.
Figure 3.2.
3. *Embryonic expression of a P2X<sub>3</sub> receptor in zebrafish*

**Figure. 3.3. legend**

*p2x3* expression in trigeminal cells and in putative lateral line ganglion cells. Dorsal (A,B,D-G with anterior to the left) and lateral (C,H,I) views of embryos showing *p2x3* expression (in blue) and HNK1 antibody (in brown) at the stages indicated. (A-C) Expression in the trigeminal ganglion (A) and the posterior lateral line ganglion (B,C). The arrowheads indicate the trigeminal ganglion and the arrows the posterior lateral line ganglia. (D-F) Double labelling of *p2x3* and HNK1 in the trigeminal ganglia. Arrows show the split domains of *p2x3* expression in the trigeminal ganglia (E). (G) Double labelling of *p2x3* and Islet-1 in the trigeminal ganglion. An arrowhead indicates a putative placode derived cell that only expresses Islet-1 and the arrow points to a double labelled putative neural crest derived cell. (H,I) Lateral views of *p2x3* expression in both a narrowminded mutant (H) in which no staining can be seen and a wild-type sibling (I) which shows normal expression. Abbreviations: o, otic vesicle; s, first somite.
Figure 3.3.
3. Embryonic expression of a P2X3 receptor in zebrafish

position occupied by the posterior lateral line ganglion (Metcalfe et al., 1985), suggesting that late p2x3 expression may be within sensory neurons of this cranial ganglion.

3.5.3. Double labelling of p2x3 positive cells

To determine the identity of cells expressing p2x3 between the eye and the otic vesicle, we combined in situ hybridisation with both HNK1 and Islet-1 antibody staining (Metcalfe et al., 1990, Korzh and Thor, 1993). HNK1 antibody labels developing neurons - initially trigeminal ganglion neurons, later Rohon-Beard neurons and eventually many CNS and PNS neurons. Double staining with HNK1 reveals that the bilateral sites of p2x3 expression are cells of the trigeminal ganglion (Fig. 3.3E), but mostly not the differentiated HNK1 expressing neurons themselves (Fig 3.3F). Islet-1 immunostaining labels the nuclei of primary neurons (Korzh and Thor, 1993). Double labelling with anti Islet-1 antibody shows that a subset of Islet-1 positive trigeminal cells coexpress p2x3 and this is consistent with the HNK1 double staining (Fig. 3.3E,F). It is known that the trigeminal ganglion is composed of both neural crest and placode derived cells (see review for chick by Noden, 1993, for mammals by Verwoerd and van Oostrom, 1979). The localisation of p2x3 transcripts to HNK1 negative cells is consistent with expression being restricted to the neural crest derived component of the trigeminal ganglion, as early differentiating neurons derive from the placodal lineage, whereas neural crest gives rise to later differentiating neurons and glia (Moody et al., 1989, D’Amico-Martel and Noden, 1983). The neural crest origin of p2x3 expressing trigeminal cells is also supported by the early lateral stripes of expression which are in the right place to be migratory neural crest that could include the cells that later express p2x3 in the trigeminal ganglion.

To further analyse p2x3 expression in the zebrafish, expression of the gene was analysed in a zebrafish mutant, narrowminded (Artinger et al., 1999). The mutation leads to a reduced number of early neural crest cells and
3. Embryonic expression of a P2X3 receptor in zebrafish

eliminates all Rohon-Beard neurons. Embryos at 24 hpf show little response to touch, reduced pigmentation and a reduction in the size of the trigeminal ganglion as only placode derived cells are present (Artinger et al., 1999). In situ hybridisation on 24 hpf narrowminded mutants shows a lack of p2x3 expression in both the trigeminal anlage and Rohon-Beard neurons (Fig. 3.3H). This confirms the identity of p2x3 expressing trigeminal cells as neural crest derivatives.

3.5.4. Functional analysis

The expression of p2x3 during zebrafish development suggests that, in a similar fashion to other species, the receptor has a sensory role during embryogenesis. Researchers in the zebrafish field have various tools and techniques that can be employed to perturb development and then assay changed phenotypes and behaviours. The next part of this chapter describes experiments that aim to elucidate the functional role of this gene.

The expression of p2x3 in the trigeminal nerve and Rohon-Beard cells (RBCs) suggests that the receptor is involved in response to light touch or tickle. Rohon-Beard cells in particular are touch receptors that are involved in an early embryonic escape response. The trigeminal ganglion is the largest sensory ganglion during development and provides sensation to the area of embryo forward of the otic vesicle. The first functional experiment was to knockout p2x3 function by the injection of a morpholino and then analyse changes in the cellular composition of the trigeminal ganglion and in the responsiveness of touch activation of the trigeminal ganglion and Rohon-Beard cells.

3.5.5. Morpholino design and injection

Morpholinos are sequence specific inhibitors of gene translation that can be injected at a single cell stage to make a knockout of the gene of interest (Ekker, 2000). They are chemically modified DNA sequences in which the ribose backbone of the DNA molecule is replaced with a morpholine moiety
3. Embryonic expression of a \( P2X_3 \) receptor in zebrafish

(Summerton \textit{et al.}, 1999, Ekker, 2000). A phosphoramide linkage is added to the moiety to return the charge on the backbone to neutral (Fig. 3.4). Morpholinos appear to give little background effects and cellular toxicity. Unlike traditional anti-sense RNA injection experiments where the mRNA is degraded by RNAse H, morpholinos act by inhibiting the initiation of translation. The mRNA is left intact but the protein has not been translated.

3.5.6. Limitations of morpholinos

Morpholinos have been shown to work with both high efficiency and can be used in combinations to produce double mutants (Ekker, 2000). The injected morphant is potently active at the beginning of development and continues to be active throughout development. However, their use is not without its limitations, many of which hold true for other forms of gene knockout. Functional redundancy of genes will mask some of the knockout effects, a problem which can be circumvented by injection of combinations of morpholinos against orthologues. Other associated problems include inability to inhibit the translation of some genes, possibly because of complex promoter structure, and failure to inhibit translation because of differences in the genetic background between morpholino and embryo. Genes with known polymorphisms (such as \textit{oep}, given as an example by Ekker, 2000) sometimes show variation in the inhibition caused by morpholino injection.

3.5.7. Morpholino against \( p2x3 \)

In order to generate zebrafish embryos which had a functional knockout of the \( p2x3 \) gene, a morpholino was designed that bound to the initiation methionine and first twenty base pairs of sequence to a morpholine moiety (Gene-tools inc.). A lack of antibody against the zebrafish gene meant that the best way to assess the viability of the knockout was to compare touch responses of morphant embryos to those of uninjected wild-type embryos.
Fig. 3.4. Chemical structure of morpholinos. The base pairs used in the construction of the p2x3 morpholino were taken from the DNA sequence immediately following the initiation methionine. Morpholino diluted in Danieau buffer (see Materials and Methods) was injected into single cell embryos and the morphology was screened at 24 hours.
3. Embryonic expression of a P2X$_3$ receptor in zebrafish

In order to make fish with no functional $p2x3$ receptors a $p2x3$ morpholino was injected into wild-type embryos at the single cell stage and the embryos were allowed to develop for 24 hours (see Material and Methods for injection protocol). The concentration of morpholino injected was titred carefully: too high a concentration and cell death and embryonic abnormalities were seen, too low a concentration and the protein translation would not be blocked. The concentration of morpholino that was injected ranged between 0.2mm and 2mM. Typically, the ideal concentration would be the maximum amount that could be injected without causing cell death. Embryos at 24 hours were screened for changes in gross morphology and then either fixed and stained using an anti-acetylated tubulin antibody, or used in a simple functional test to assess the touch responsiveness of the embryo.

3.5.8. Phenotype of morphant embryos

Morphology screening of the injected embryos showed that they appeared to develop normally, with no phenotypic abnormalities visible to the eye under a dissection microscope (Nikon). The embryos proceeded to develop normally until hatching stage and were then presumed to have been morphologically unaffected by injection of the morpholino (Fig 3.5.).

3.5.9. Tubulin staining of morphant embryos

Anti-acetylated tubulin antibody binds to protein expressed in all developing neurons in the zebrafish embryo (Wilson et al., 1990) and so provides a good marker to study changes in the developing axon scaffold. Anti-Hu antibody marks differentiated neural cell bodies (Dalmau et al., 1991). Antibody staining of morphant embryos showed no changes when compared to wild-type uninjected embryos. The trigeminal ganglion of the developing zebrafish embryo is a very complicated structure with many cell bodies and axons. They develop as a wedge-shaped network of randomly crossing neurons rather than in a stereotyped pattern. As such, analysis of morphant
Fig. 3.5. Photographs of wild-type and p2x3 morpholino injected embryos at 24hpf. The injected embryos show no differences when compared to uninjected controls.
3. Embryonic expression of a P2X<sub>3</sub> receptor in zebrafish

embryos could only aim to look for a significant reduction in the size of the ganglion instead rather than an absence or misplacement of single neurons.

The injected morphant embryos appeared to show no change in size or complexity of trigeminal ganglion (Fig 3.6.). The Rohon-Beard cells were not counted, but as both cell types are thought to come from a common origin, the migrating neural crest, there is no reason to believe that the Rohon-Beard cells (RBCs) would show change when the trigeminal ganglion did not.

3.5.10. Patch clamping of fish trigeminal ganglia

In order to compare the response traces to agonist application, an attempt was made to patch clamp cells of the trigeminal ganglion from both control uninjected and morphant embryos. Several papers have described techniques for patch clamping neurons in vivo in the zebrafish (Drapeau et al., 1999, Ribera and Nüsslein-Volhard, 1998). The papers have concentrated on patch recordings from RBCs and other sensory motorneurons. Repeated attempts to mount zebrafish embryos and to patch the cells of the trigeminal ganglion were not successful. The glue used to mount preparations described in other papers did not work in our preparations and the patch apparatus that we used made it difficult to get recordings from the cells. RBCs are smaller than trigeminal ganglion cells and so would have been more difficult to patch in a live embryo preparation. Alternative methods of testing the function of the receptors and the morpholino were devised.

3.5.11. Touch responsiveness of injected vs uninjected embryos

Dishes were prepared by glueing a glass slide to the inverted lid of a petri dish. This provided a small "shelf" against which an embryo could be positioned in a small amount of water. In order to provide a touch stimulation to the embryo which was both gentle and controlled, a von Frey hair was attached to a micromanipulator. Following stimulation, the embryo in the petri dish flicked its tail and tried to push away from the slide. This action allowed the embryo to
Fig. 3.6. Antibody staining on wild-type control (A,C) and P2X3 morpholino injected (B,D) embryos. Injection of the morpholino appeared to cause no changes in the morphology of the trigeminal ganglion. (A) and (B) show anti-acetylated tubulin antibody staining in brown (arrowheads) and (C) and (D) show anti-Hu antibody staining in brown (arrowheads). All panels show anterior to the left and dorsal to the top. Embryos are 36 hours old.
move away from the vicinity of the von Frey hair. Knock-out embryos were made by injection of p2x3 morpholino at the single stage cell to inhibit the transcription of the protein product. Stimulation with a von Frey hair produced a similar result in both injected and control embryos (Fig. 3.7 and Fig. 3.8.). The morpholino did not appear to be inhibiting the response to tactile stimulation.

**Touch responsiveness of embryos incubated in P2X₃ blockers**

The next question that was addressed asked whether the lack of change in touch response following morpholino injection was due to the inability of the morpholino to block translation or to a lack of involvement of the receptor in mediating the touch response. Zebrafish embryos were incubated in solutions of known purinoceptor blockers, at concentrations that should inhibit the activity of the receptor. Two time courses of receptor block were tried; 2 hours and 24 hours. The embryos were then placed in a petri dish again and stimulated using a von Frey hair attached to a micromanipulator. Video clips were made of the response to tactile stimulation and no differences were seen between embryos incubated in antagonists and controls (Fig. 3.9.). The experiments were repeated using five or six different embryos. Once the response type and duration had been established, three separate films were made to confirm the response and for further analysis. The antagonists that were used included: suramin, 10⁻⁴M; PPADS, 10⁻⁴M; TNP-ATP, 10⁻⁴M and Reactive Blue 2, 10⁻⁴M.

**3.5.13. Single dose application of agonist causes an escape response**

The next experiment tested the ability of P2X₃ receptors to respond to a single large dose of agonist. The agonist was presented as a large bolus from a Gilson pipette, at a concentration many times larger than physiological levels. The agonists used in the experiments included ATP, ADP and α,β meATP. Application of ATP to a wild-type embryo caused a large sensory response. The embryos thrashed their tails from side to side in order to try and move away from the source of the stimulation, an escape response. Application of water (as a control) on the other hand caused no response. Responses were measured
Fig. 3.7. Frames taken from a film of wild-type embryos stimulated with a von Frey hair. The embryo displays a stereotyped escape response and uses tail flicks to move away from the hair. This excerpt lasted less than one second. The whole movie is submitted as a quicktime file attached to the back of the thesis.
Fig. 3.8. von Frey hair stimulation of $p2x3$ morpholino injected embryos. The response is no different to that of the uninjected controls, an escape response is seen. This excerpt lasted less than one second. The whole film has been submitted as a quicktime file on a CD attached to the back cover of the thesis.
Fig. 3.9. von Frey hair stimulation of embryos incubated in a P2 receptor blocker. This time the antagonist was PPADS. The duration of the response was under one second. The whole film is submitted as a quicktime file on a CD attached to the back cover of the thesis. Other antagonists were also tested and the results are included on the CD.
using a video camera and dissection microscope (Fig. 3.10., water application; Fig. 3.11., ATP application).

3.5.14. Application of other agonists caused no escape response

The application of other P2X₃ receptor agonists, including ADP, α,β meATP and adenosine was tested, to see if the escape response was restricted to ATP application. No other agonist was able to produce the escape response seen by ATP application (Fig. 3.12., Fig. 3.13., response to α,β meATP not shown). The ATP used in this experiment was measured to have a pH value of 4 and in order to remove the possibility that the escape response was due to the pH of the applied solution, a buffer was made from a pH 4.0 buffer tablet. Application of the pH 4 solution elicited no escape response (Fig. 3.14.). This suggests that the response was due to the ATP molecule and not the pH of the ATP solution.

3.5.15. Application of agonist to embryos incubated in P2 antagonists

In order to show that responses seen when embryos were given a single large dose of ATP were indeed due to P2X₃ receptor activation, embryos were incubated in antagonists for either 2 hours or 24 hours and then given a single bolus dose of ATP. Response were measured using a video camera and dissection microscope. Incubation of embryos in antagonists for long periods of time appeared to have no effect on the response to ATP. Incubation of embryos in antagonists for two shorter periods of time (either 30 minutes or 10 minutes) also elicited no change in the response to ATP application. A large escape reaction, with severe tail flicks to either side was seen (Fig. 3.15.). P2 receptor antagonists that were used in this experiment included PPADS, and suramin. The antagonists were used at a concentration of 30 µg/ml

3.6. Discussion

3.6.1. Expression of p2x3 in fish
Fig. 3.10. Application of water to a wild-type embryo using a pipette. The embryo did not respond. The frames here last for around one second, but the embryo remained still for longer than the duration of the film. The whole film has been submitted as a quicktime file on a CD attached to the back cover of the thesis.
Fig. 3.11. Application of an ATP solution to a wild-type embryo using a pipette. The embryo clearly responds by thrashing the tail from side to side. The frames shown here last for about one second, although the response continues for a lot longer. The whole film is included as a quicktime file on a CD attached to the back cover of the thesis.
Fig. 3.12. Excerpts taken from a film of an ADP injection onto wild-type embryos. No response is seen to the ADP application. The whole film is submitted as a quicktime file attached to the back of this thesis.
Fig. 3.13. Film showing injection of adenosine onto a wild-type embryo. The embryo does not respond by moving its tail. The only movement comes from the injected adenosine solution. The whole film is submitted as a quicktime file attached to the back of the thesis.
Fig. 3.14. Excerpt from film showing injection of a pH4 solution onto wild-type embryos. No response is seen to the addition of the solution. This suggests that the response to ATP is not caused by its pH level. The whole film is submitted as a quicktime file attached to the back of the thesis.
Fig. 3.15. Excerpts from a film showing an injection of ATP onto an embryo that has been incubated in a P2X3 receptor antagonist, TNP-ATP. The entire sequence lasts about one second and is submitted as a quicktime file on the back cover of this thesis. The antagonist does not appear to block the embryo's response to ATP.
3. Embryonic expression of a P2X₃ receptor in zebrafish

The zebrafish p2x3 gene was found to be expressed in the neural crest derived cells of the trigeminal ganglion and in Rohon-Beard cells. Both these cell types have a function as touch sensitive receptors active during early zebrafish development. The expression of p2x3 in these cells is consistent with evidence found in higher vertebrates. Evidence from other species suggests that the P2X₃ receptor is involved in signal transduction in sensory ganglia (Tsuda et al., 1999) and in the micturation response to bladder filling (Cockayne et al., 2000, Vlaskovska et al., 2000). However, no expression was seen in other brain areas or the bladder of the zebrafish.

3.6.2. A suggested common origin of p2x3 expressing cells

The earliest expression of p2x3 was visible as two stripes of expression between the developing eye and ear, lateral to the anterior neural plate tissue (Fig. 3.2B). These stripes appear to correlate with the migratory population of neural crest cells, that move posteriorly down the embryo and form a wide variety of cell types. Cranial neural crest proceeds to differentiate and form among other things branchial arch tissue, melanocytes and sensory ganglia of the head. Interestingly, there is some evidence to suggest that Rohon-beard cells are also neural crest derivatives, suggesting a common origin of p2x3 expressing cells. Analysis of the narrowminded mutant revealed that embryos lacking a population of early migrating neural crest cells also lacked Rohon-Beard cells (Fig. 3.3H). At 24hpf, narrowminded embryos were less responsive to touch than wild-type siblings. The mutant recovered later on during development and showed normal sensory responses, as late developing crest cells replaced the missing early migratory cells. By 48hpf the response to touch stimulation was normal (Artinger et al., 1999). Further evidence for the common origin of these two cell types was presented in a paper by and Cornell and Eisen (2000). Zebrafish embryos with a point mutation in the delta A gene were seen to have supernumery numbers of Rohon-Beard cells and reduced expression of trunkal neural crest derivatives. Analysis of an equivalence group in the zebrafish trunk and tail neural plate showed that default cell type during
3. Embryonic expression of a P2X₃ receptor in zebrafish

development was the Rohon-Beard cell. Rohon-Beard cells developed as a central cell, surrounded by cells that would proceed to form other trunkal neural crest derivatives (Cornell and Eisen, 2000). Loss of the delta A gene in the mutant upset the normal Notch-Delta signalling pathway and resulted in an increased number of cells developing into Rohon-Beard cells, instead of being switched to a neural crest lineage.

These two pieces of evidence suggest that the expression of p2x3 in the zebrafish embryo may well be seen in two cell types derived from an early equivalence group, present at the lateral edge of the neural plate.

3.6.3. Species differences between expression patterns of p2x3

Comparison with the receptor expression in other species shows two marked differences: zebrafish p2x3 is expressed neither in other brain nuclei nor the gut. There are several possibilities that could account for this altered difference in expression. Studies on the P2X₃ receptor in other species often look at late or post-natal development. Expression in the fish was examined until larval stage, when the fish is no longer considered to be embryonic. Later - in situ hybridisations may show staining in these areas but expression of a gene at such a late stage of development is unlikely to have a role in the specification of cell types forming the tissue. Post-embryonic gene expression would be much more likely to have a role in the normal function of the tissue and although the function would be undoubtedly important, it is not within the aims of this thesis to characterise late functions.

3.6.4. Genetic Orthologues in the zebrafish

The zebrafish is a modern teleost, and is thought to have undergone an extra genome duplication compared to other vertebrate species. It has been suggested that one genome duplication event occurred before the divergence of ray-finned fishes, and a second one after this event. (Postlethwait et al., 1998). Other evidence, however, argues that because various vertebrates have
3. Embryonic expression of a P2X<sub>3</sub> receptor in zebrafish

several HOX clusters, two rounds of duplication occurred before the origin of jawed fishes (Van de Peer <i>et al.</i>, 2001, Holland <i>et al.</i>, 1996). Whenever the event occurred, the result is that for every developmental gene found in other species, the fish has a potential two or three copies. Another p2x3 orthologue may exist in the zebrafish. So far, neither the Washginton EST database (http://www.wuzgr.edu) or the Sanger centre database (http://www.sanger.ac.uk) have published sequence for more than one possible P2 gene, and there has been no evidence yet for P2 orthologues in any species. However, orthologous genes often have overlapping expression patterns, a characteristic that can account for the large amount of redundancy of function seen in fish genes and which could account for p2x3 expression being more restricted than expected.

The p2x3 gene is clearly expressed in sensory cells that are activated by light touch and tickle. The trigeminal ganglion is the largest ganglion in the developing fish embryo, sensory for the area of the head anterior of the otic vesicle (Metcalfe <i>et al.</i>, 1990). Rohon-Beard cells, on the other hand, are the largest and most dorsal neural cells in the developing trunk. They form fascicles that ascend and descend the nerve cord, forming an embryonic neural network (Metcalfe <i>et al.</i>, 1990). Numerous small axons connect the cell to the skin and allow activation by tickle or light touch. Both cell types are thought to be implicated in an escape response following activation. Touching a zebrafish embryo at 24hpf or later causes a tail flick from side to side in an attempt to move away from the source of the touch. The response is thought to have been developed to allow amphibious embryos to survive attack during early life. The removal of Rohon-Beard cells by programmed cell death to be replaced by dorsal root ganglion cells (DRGs) highlights their role as a sensory cell which is solely active during early development. Rohon-Beard cells are only found in amphibians. Presumably, animals born on land either develop inside of the mother (mammals) or in a protective environment such as an egg where the
3. Embryonic expression of a P2X3 receptor in zebrafish

need to move away is not so great.

3.6.5. Similarity between zebrafish and other species

Both expression of the zebrafish gene and the published data on P2X3 receptors in other species show that the receptor is involved in a sensory response of some kind - be it in the pain pathway or in an escape response to touch or tickle. In this way the role of the receptor can be seen to be fairly conserved throughout evolution.

3.6.6. Pharmacological differences

The zebrafish receptor has been shown to act as a heteromultimer with the P2X2 receptor and has both the fastest activation and kinetics of desensitisation of any P2X receptor studied so far. Our suggested role of p2x3 in a rapid response to touch fits in well with these channel opening kinetics. Surprisingly the zebrafish P2X3 receptor activation lasts for a very long time (Egan et al., 2000). This would suggest that after responding to activation by thrashing the tail and moving away, the embryo was unable to respond further. Analysis of films showing the response to touch by a von Frey hair (and indeed any person who has ever dechorionated an embryo with forceps) shows that this is clearly not the case. Repeated poking of an embryo with a von Frey hair caused repeated responses.

There are several reasons why the embryo would be able to respond to rapid stimulation despite other predictions from the receptor pharmacology. In vivo the receptor has been shown to act as a heteromultimer, combined with the P2X2 receptor (Egan et al., 2000). The desensitisation of this receptor is slower and lasts for a shorter period of time. Secondly, repeated activation of the channels in the embryos may not necessarily cause activation of the same receptor. Desensitisation may be localised and allow the next nearest receptor to respond to stimulation. It is not unusual to find differences between in vivo and in vitro responses.
3. Embryonic expression of a P2X3 receptor in zebrafish

The use of anti-sense oligonucleotides to block the rat P2X3 receptor has been published (Dorn et al., 2001). These oligonucleotides may circumvent some of the difficulty of pharmacological characterisation of P2 receptors caused by a lack of specific antagonists.

3.6.7. Evolutionary aspects

There have been few studies published so far about the expression and function of P2 receptors in lower vertebrates. Analysis of different species can give important information about the underlying biophysical and pharmacological properties of this receptor family.

The analysis of paralogues in different species gives the opportunity to find out how evolutionary changes can be measured at the molecular level. The zebrafish P2X3 receptor is more divergent than those of other species. It shows 54% homology to the nearest paralogue (the rat P2X3 receptor) whereas other species show much higher homologies. These changes can go some way to explaining the large pharmacological differences between the fish and other species. Several pieces of evidence have shown that changes in the underlying amino acid sequence of a receptor will change the binding affinity of agonists and antagonists. A good example of this is the P2X4 receptor, which is insensitive to block by PPADS and suramin. Changing the glutamate residue at position 249 to a lysine residue will allow the antagonists to bind and block the receptor (Buell et al., 1996). This will be discussed in greater detail in the next chapter. Although the links between amino acid sequence and ligand binding affinity are intriguing, a certain caveat must be mentioned. The zebrafish homologue is extremely divergent and it is too simple to claim that this divergence simply causes a change in pharmacology.

3.6.8. Analysis of the function of p2x3

One of the underlying aims of this thesis was to look at the function of P2
3. Embryonic expression of a P2X3 receptor in zebrafish

receptors during development as well as cloning and looking at the expression. Evidence from the chick P2Y1 and Xenopus P2Y8 receptors suggested that functional studies would provide interesting data. One of the difficulties with the zebrafish p2x3 gene was to find a functional test that was suitable to show the underlying function of the receptor. Several techniques were tried to assay the function. Touch responses to von Frey hairs and stimulation by application of a bolus of agonist were found to be the most successful tests.

3.6.9. Morpholino injections

Injection of a p2x3 morpholino appeared to show no phenotypic or functional changes to the development of the embryo. There are several reasons why this may have occurred. The promoter region of the gene may be complicated and so not enable the morpholino to bind successfully and inhibit translation. The gene may also have multiple leader sequences or be polymorphic and show allelic differences (Ekker et al., 2000). It is unlikely that the morpholino itself does not bind to an area of the gene that would inhibit translation, as the morpholino was designed against the amino acids directly following the initiation methionine. The absence of an antibody against the receptor protein means that it was difficult to directly assess whether the morpholino was effective in reducing protein levels.

3.6.10. Stimulation with a von Frey hair

Stimulation of either trigeminal ganglion cells or Rohon-Beard cells with a von Frey hair provoked a stereotyped escape response. Neither injection of a morpholino nor incubation in antagonists at concentrations that usually blocked responses in other organisms, inhibited this response. One explanation that could account for this is that the morpholino and antagonists both fail to block the activation of the receptor. One possibility that would account for a lack of antagonist block is that the antagonist is not able to reach the receptor. A way to confirm this would be to see if an agonist can activate the receptor.
3. Embryonic expression of a P2X3 receptor in zebrafish

3.6.11. Stimulation with ATP

Application of a large dose of ATP applied directly to the embryo caused a large scale response. The embryo moved its tail vigorously for the duration of the film and continued after the video camera stopped recording. The end of the response was presumably delayed until the ATP degraded and no longer activated the receptor. The dose used in this experiment was far larger than any physiological dose and we do not pretend that the experiment was carried out under physiological conditions. Injection of water produced no effect. This showed that the ATP solution was activating the receptor and not effecting the embryo in another way.

Application of either a solution with a similar pH to ATP, ADP, adenosine or α,β meATP also caused no escape response to be recorded from the embryo. This result is surprising if the ATP is indeed stimulating a P2X3 receptor: the pharmacological profile of the zebrafish P2X3 receptor has been published and shows that when expressed in *Xenopus* oocytes, zebrafish P2X3 is activated equipotently by ATP and ADP. α,β meATP only acts as a weak agonist. The changed response that I have recorded may be due to differences in the receptor activation *in vivo* as compared to an *in vitro* system, or may be due to the activation of more than one subtype of P2 receptor by the ATP in the films.

In a similar way to results seen in the experiment using a von Frey hair to stimulate antagonist treated embryos, incubation in an antagonist failed to block the response to agonist application. As ligands can reach the receptor, an explanation may be that the antagonists are not able to bind and block the receptor or that the antagonists have degraded under experimental conditions and are no longer potent. The incubation time used for the antagonists was comparatively long - either 24 hours or 2 hours. The long incubation may have allowed the antagonist to be broken down and the receptor to recover from the blocking. Shorter incubation times of antagonist were also used, to try and block
3. Embryonic expression of a P2X₃ receptor in zebrafish

the stimulation by ATP. Incubation times of either 10 minutes or 30 minutes were not able to block the response to ATP.

Alternatively, P2X₃ is not the only receptor present in the sensory ganglia at this stage and other receptors may also be responding both to touch and agonist application. One possibility would be that ATP activates P2 receptors and an unknown receptor subtype at the same time, but the antagonists can only block the P2X₂ or P2X₃ receptors. Perhaps following incubation in antagonists, the unknown receptors can still respond to either ATP application or von Frey hair stimulation.
4. Characterisation of a $P_{2X4}$ receptor in zebrafish

CHAPTER FOUR

Characterisation of $P_{2X4}$ receptor expression in zebrafish

4.1. Abstract

From studies in other species it is known that the $P_{2X4}$ receptor is expressed in non-neuronal tissues including blood vessels, bladder, heart, thyroid, lung, testis, vas deferens, stomach, placenta, osteoclasts, prostate, B lymphocytes, colon and islet cells of the pancreas and it is the only purinoceptor to be found in acinar cells of the salivary gland (Ralevic and Burnstock, 1998). It is a predominant receptor in the CNS along with $P_{2X2}$ and $P_{2X6}$. The channel has been shown to function in synaptic transmission in the central nervous system. However, little is known about the expression and function of the $P_{2X4}$ receptor during embryonic development.

This chapter describes the expression of a novel gene ($p2x4$) encoding a $P_{2X4}$ receptor during zebrafish development. The expression is seen in the gut of the fish from about two and half days of development. The expression of $p2x4$ persists throughout larval development and is still present in adult tissue. Possible functions for the receptor are discussed.

4.2. Introduction

$P_{2X4}$ receptors have been cloned from a wide variety of species and the pharmacology and functional roles have been published (North and Suprenant, 2000). The first part of this chapter will briefly describe the pharmacology and tissue distribution of this receptor in other species. A more complete description of the pharmacology and tissue distribution of the $P_{2X4}$ receptor is included in the general introduction in chapter 1. The fish homologue has an expression pattern that is similar, but not identical, to that of other species. Comparison to
4. Characterisation of a P2X\textsubscript{4} receptor in zebrafish

P2X\textsubscript{4} receptors in other species suggests conserved functional roles for the receptor.

4.3. Pharmacology of the P2X\textsubscript{4} receptor

The P2X\textsubscript{4} receptor has been cloned from a variety of tissues including rat brain, hippocampus, dorsal root ganglion cells and endocrine tissue (Buell et al., 1996, Séguéla et al., 1996, Soto et al., 1996, Bo et al., 1995). The pharmacology of the receptor has been studied by expression of rat or human genes in *Xenopus* oocytes (Garcia-Guzman et al., 1997, Bo et al., 1995). The channel shows a wide range of pharmacological properties including pH sensitivity, modulation by metal ions and a lack of inhibition by P2 receptor blockers (Wildman et al., 1999a, Garcia-Guzman et al., 1997, Bo et al., 1995). The P2X\textsubscript{4} receptor is potently activated by 2MeSATP, but α,β Me-ATP is weak or inactive. P2X\textsubscript{4} receptors are not antagonised by either suramin or PPADS as a crucial lysine residue that allows the binding of antagonists in other subtypes is missing. Agonist binding, however, is unaffected (Buell et al., 1996). Agonist responses have been found to be potentiated by allosteric regulation by either ivermectin or reactive-blue2 (Khakh et al., 1999, Miller et al., 1998). Many of the properties of the P2X\textsubscript{4} receptor have been assigned to key amino acids in the extracellular domain of the receptor. Mutagenesis of these amino acids changes the pharmacological profile of the channel, properties that provide important regulation of channel function. These studies of the P2X\textsubscript{4} receptor have been influential in defining important amino acid sequences and residues that control the binding affinities of both agonists and antagonists to P2X receptors (Clarke et al., 2000, Carpenter et al., 1999). Analysis of differences between both species and receptor subtype allows the variation in pharmacology between paralogues to be studied. These variations can then be attributed to changes at the molecular level.

The pharmacology of the zebrafish P2X\textsubscript{4} receptor has not been reported in the literature and it is not within the aims of this thesis to undertake
4. Characterisation of a P2X4 receptor in zebrafish

pharmacological characterisation. A review of data from other species is included as part of the introduction in chapter 1.

Studies comparing P2X3 receptor pharmacology of other species to that of the zebrafish homologue (Boué-Grabot et al., 2000) have shown that it is not possible to accurately predict the properties of the zebrafish receptor. The high degree of divergence of P2X4 receptor amino acid sequences during evolution means that is extremely likely that pharmacological properties will be changed in different species.

4.4. Molecular characteristics of P2X4

The P2X4 receptor has been shown to have two transmembrane domains, with an extracellular joining loop and intracellular C and N terminals. P2X4 has also been shown to form both heteromultimers with the P2X6 receptor (Lê et al., 1996) and splice variants (Carpenter et al., 1996, Townsend-Nicholson et al., 1996).

4.4.1. Splice variants

Several species have been found to have P2X4 receptor splice variants expressed in different tissues of the animal (Carpenter et al., 1999, Townsend-Nicholson et al., 1999). An example of these are the variants found in human brain tissue. Splice variants can be formed by either deletion or addition of a number of amino acids from the extracellular domain of the receptor. The splice variants in human brain were studied using clones expressed in Xenopus laevis oocytes to show that the pharmacological properties had changed (Carpenter et al., 1996). A second study compared differences between murine splice variants (Townsend-Nicholson et al., 1999). The first variant (mP2X4) was 388 amino acids long and showed homology of 94% to the rat P2X4 receptor. The second clone was named mP2X4a and lacks a 27 amino acid cassette that corresponds to exon 6 of the gene. Expression in Xenopus oocytes showed an altered pharmacology between the two variants. The P2X4a receptor was only
4. Characterisation of a P2X<sub>4</sub> receptor in zebrafish

able to form poorly functional homomultimeric receptors. In combination with mP2X<sub>4</sub> a heteromultimeric receptor was formed that exhibited reduced affinity to ATP (Townsend-Nicholson et al., 1999).

In a similar way to the changing of receptor subtypes, changing the splice variant expressed in any tissue allows different responses to be seen from application of the same agonist. During evolution, splice variants of the P2X<sub>4</sub> receptor may have evolved to allow a greater number of responses to ATP to be seen in different tissues of the same animal.

4.4.2. Promoter analysis

Studies of P2X<sub>4</sub> receptor expression in human umbilical vein endothelial cells (HUVECs) have shown that following shear stress to the endothelial cells, the expression levels of the receptor are decreased (Korenaga et al., 2001, Yamamoto et al., 2000). Shear stress in HUVECs is caused by blood flow through the vein. Injection of anti-sense nucleotides designed against the P2X<sub>4</sub> receptor abolished the Ca<sup>2+</sup> ion influx measured following shear stress. Conversely, transfection of HEK cells with P2X<sub>4</sub> conferred the cells the ability to produce a shear stress dependant Ca<sup>2+</sup> influx (Yamamoto et al., 2000). Analysis of the promoter region of the gene has shown that there is a 131 base pair sequence around the initiation methionine (from -112 to +19 bp around the transcription start site) that contains an Sp1 transcription factor binding site. This transcription factor is critical for the responsiveness to shear stress (Korenaga et al., 2001). Mutation of the Sp1 site reduced the ability of the P2X<sub>4</sub> promoter to respond to changes in shear stress levels.

4.5. Results

An EST clone that showed high homology to P2X<sub>4</sub> receptors of other species when compared using a BLAST (Basic Local Alignment Search Tool) was obtained from the Washington University EST database (www.wuzgr.edu). The complete sequence for the zebrafish P2X<sub>4</sub> receptor has been published and
4. Characterisation of a P2X4 receptor in zebrafish

has been assigned the Genbank accession number AF317643. p2x4 has an open reading frame of 1170 base pairs and codes for a protein of 390 amino acids (Fig. 4.1.). The receptor appears to have a similar predicted structure to other P2X family members, with two transmembrane domains and extracellular amino- and carboxyl- terminals.

4.5.1. Tree diagram to compare homology

The homology of P2X4 receptor paralogues from six different species were compared using a Jotun Hein alignment program (Hein, 2001) with the zebrafish sequence as a consensus (Fig. 4.1). The fish sequence shows some areas of high homology that may represent the transmembrane domains (Fig. 4.1., amino acids 31 - 53 and 338 - 364). These domains are fairly highly conserved and agree with the transmembrane domains found by comparing all rat P2X4 sequences (Soto et al., 1995). The highest homology is seen to the *Xenopus* P2X4 sequence with a 57.7% similarity between the sequences. This degree of similarity is low: between chick and *Xenopus* the similarity is 70.1% and it is 93.4% between rat and mouse. A similarity tree, based on amino acid homology was constructed. The tree confirms that the zebrafish sequence shows high homology to the *Xenopus* paralogue (Fig. 4.2.). Mammalian genes are also extremely closely related. p2x4 genes are also seen to show high similarity to p2x1 and p2x7 genes. For Genbank accession numbers please refer to the figure legend (Fig. 4.2.).

4.5.2. Wholemount in situ hybridisation pattern

The first expression of the p2x4 gene was detected at around two and a half days of development. Analysis of embryonic stages before this showed no expression.

From two and a half days of development onwards, the gene was seen to be expressed in tissue of the stomach and gastrointestinal tract (GIT, Fig. 4.3., arrowhead in B,C and D). A large area of staining was seen in the stomach, an
Fig. 4.1. Jotun Hein alignment of P2X4 receptor homologues from several different species. The fish clone shows highest sequence identity to the frog clone. Black shading shows exact match to fish sequence. Genbank accession numbers: human, NP00251; rat, S62359; mouse, AAC95601; chick, AAD01645; frog, AAG45104; fish, AAK00945. Alignment produced using SeqMan software, part of DNAstar package. Putative transmembrane domains are underlined in grey and labelled M1 and M2.
Fig. 4.2. Phylogenetic tree comparing homology of all cloned P2X receptors. The tree is based on a Jotun Hein alignment, which provides the best resolution. The zebrafish P2X4 receptor shows highest homology to other P2X4 genes. Genbank Accession numbers: human x1, P51575; rat x1, P47824; mouse x1, P51576; human x2, AAD42947; guinea x2, O70397; rat x2, P49653; human x3, P56373; rat x3, P49654; human x4, NP002551; rat x4, S62359; mouse x4, AAC95601; chick x4, AAD01645; human x5, Q93086; rat x5, CAA63052; human x6, O15547; rat x6, P51579; mouse x6, O54803; human x7, Q99572; rat x7, Q64663; mouse x7, CAA08853
4. Characterisation of a P2X4 receptor in zebrafish

Figure 4.3. legend

*p2x4 in situ* hybridisation staining on wholemount zebrafish embryos. *In situ* staining is seen in blue. All embryos are shown with anterior to the left. (A, B, C, D) are lateral views. (E, F, G H) are ventral. Embryos were staged at three days, the earliest expression of *p2x4* (A, C, E, G). Arrowheads mark staining in the stomach and gut. Arrows show staining in the brain ("B") and notochord ("N"). Staining was present in the same tissues at 5 days (B, D, F, H). Arrowheads show staining in gastrointestinal tract. Ventral views at both stages (3 days (E) and 5 days (F)) show that the gastrointestinal tract is not centrally located but is displaced to one side (arrowheads). (G) and (H) show staining in the hindbrain, optic commissure ("O") and posterior commissure of the developing brain ("PC"). Once again, staining is seen at 3 days (G) and 5 days (H). Weak staining is also present in one of the cell layers of the retina (panel G, "R") of both 3 day and 5 day larvae.
Abbreviations: B, brain; N, notochord; R, retina; O, optic tract; PC, posterior commissure; 3d, 3 days post fertilisation; 5d, 5 days post fertilisation.
Characterisation of a P2X<sub>4</sub> receptor in zebrafish

...an oval shaped piece of tissue that was ventral to the otic vesicle and first somite. The tissue was completely covered by, but not composed of, yolk cells (Fig. 4.3A). Rough dissection to remove the overlying yolk cells from the \textit{in situ} labelled tissue caused dissagregation of the tissue and made analysis of the intact wholemount p2x4 positive tissue difficult. Staining was also seen in the gastrointestinal tract dorsal to the yolk extension. The expression was expressed the length of the extension, ending the cloaca (Fig. 4.3A, B). Ventral views of embryos show that the p2x4 positive tissue was not centrally located but instead is displaced to the right hand side of the embryo (ventral view, Fig. 4.3E and 4.3F, anterior to left).

Weak staining was also seen in the notochord (4.3A, arrow marked N) and in some tracts of the developing hindbrain (Fig. 4.3 panels G and H). In the brain the staining appeared to be in the posterior commissure (PC) and optic tract (O). \textit{In situ} hybridisation on embryos at later developmental stages showed that the expression pattern in the notochord and gastrointestinal tract on p2x4 had not changed. Expression in the hindbrain was more variable. p2x4 was detected in the hindbrain at both three and five days of development. Weak expression of p2x4 is also present in one of the cell layers of the retina (Fig. 4.3G, R). These areas of expression are consistent with reports of P2X<sub>4</sub> receptor expression in other species.

In situ hybridisation on cryo-sections of embryos

In order to better analyse the tissue that stained positive for p2x4 expression in zebrafish larvae, \textit{in situ} hybridisation was performed on frozen sections of larvae at various stages of development. The sections allow better permeabilisation of the \textit{in situ} hybridisation probe and reveal new areas of gene expression.

p2x4 \textit{in situ} hybridisations were performed on cryo-sections of one month old fish (Fig. 4.4.). All sections are orientated with anterior to the left and
Figure 4.4. legend

In situ hybridisation staining of the p2x4 gene on one month old sections of zebrafish larvae. In situ staining is shown in blue. All sections are shown with anterior to the left and dorsal at the top. Transverse section (A) of one month larvae showing the dorsal half of the section. p2x4 is localised to the cell layer lining the gut. Increased magnification (20X, area marked by black box (B)) shows that the gut contents do not stain positive and the blue staining is localised to the cells forming the gut wall. (C) a few cells of the notochord are also p2x4 positive. Sagittal section of whole larvae (D) showing staining in stomach and the whole length of the gut tube. The arrowhead marks blue in situ staining. (E,F) high power view (10X, E and 20X magnification, F) of jaw of larvae. Blue p2x4 positive cells are seen in the jaw (marked "J") and branchial arches (marked "B"). High power views of the stomach and stomach wall (G,H) show that the stomach contents (arrow) stain blue as well as cells in the wall of the stomach (arrowhead). In a similar way to the gut, staining in stomach wall (H) is seen in all cell types.
Figure 4.4.
4. Characterisation of a P2X4 receptor in zebrafish

dorsal to the top. Transverse sections through the larvae show expression in the notochord and gut wall (Fig. 4.4A). Staining of the frozen sections also shows gene expression in the wall of the stomach and gut (sagittal sections, Fig. 4.4B, 4.4D, 4.4G and 4.4H). Some staining is also seen in the food content of both the stomach and gut (Fig. 4.4G). Analysis of transverse sections of the fish show staining in cells of the notochord (Fig. 4.4B), branchial arches (B) and jaw (labelled J, Fig. 4.4E and 4.4F). The lack of detection of these structures in wholemount in situ hybridisations is probably due to the difficulty of getting a probe to permeabilise tissue in intact embryos. The low level of staining intensity in cells of the notochord (Fig. 4.4C), branchial arches and jaw means that expression in wholemount embryos would be weak. No expression of P2X4 is seen in either the brain or the retina of one month old larvae, suggesting that the expression levels change with time.

4.5.4. In situ hybridisations on adult tissues

The gastrointestinal tract of adult zebrafish has not been well characterised. There are several good laboratory manuals available describing the anatomy of adult fish, though the information tends to be generalised to cover as many species as possible (for example, see "The handbook of experimental animals. The laboratory fish ", Ostrander, editor). There are a wide number of variations between each family of fish, even between each subtype of the order Danio. This makes it difficult to identify the tissue expressing P2X4 during development. In situ hybridisations were performed on pieces of tissue taken from an adult zebrafish (aged one year or more) to see if P2X4 expression was still present. Gene expression was detected on tissue of the gastrointestinal tract (including the stomach), whereas the liver remained clear and therefore acted as a control tissue (data not shown). This confirms that gene expression is indeed restricted to the gastrointestinal tract but not the liver. Expression in these tissues is probably seen during the whole lifetime of the mature fish, from larval stages onwards.
4. Characterisation of a $\text{P2X}_4$ receptor in zebrafish

4.5.5. In situ hybridisation on zebrafish gut mutants

The first Tübingen mutant screen identified three different zebrafish that have mutations in genes important for correct GIT development: *faust*, *bonnie and clyde* and *casanova*. The mutants were found as part of a screen for heart defects. *bon*, *fau* and *cas* all show varying degrees of cardia bifida (Stainier et al., 1996).

*bonnie and clyde* shows the least severe gut phenotype of the three mutants. *bon* has been cloned and has been shown to be a Mix family homeodomain protein. The gene is essential for early endoderm formation (Kikuchi et al., 2000). The phenotype of *bon* mutant embryos is variable - later developing gut tissue is seen in some embryos, although early tissue is always absent. *In situ* hybridisations with a $\text{p2x}_4$ probe show that brain and notochord expression is fine (Fig. 4.5.). $\text{p2x}_4$ expression in the gut is either not present at all (4.5C and 4.5D), or is seen in only a few small areas (4.5E and 4.5F). These areas correspond to late developing gut tissue.

*faust* embryos also show small areas of $\text{p2x}_4$ staining in gut tissue (Fig 4.6B and 4.6D). Expression in the brain and notochord is the similar in both wild-type and mutant (Fig. 4.6A, 4.6B, 4.6C, 4.6D, marked B and N). In *fau* embryos, a small amount of staining is seen at the anterior end of the yolk ball (Fig. 4.6B and 4.6D, arrowheads). The *fau* zebrafish locus has been shown to encode GATA5 (Reiter et al., 2001, Reiter et al., 1999) and *fau* mutant embryos show variable gut morphogenesis. Anterior gut morphogenesis is disrupted and the posterior gut varies from being absent to being present but looped in the wrong direction (Reiter et al., 1999).

*cas* is the most severe of the three gut mutants. It lacks endoderm entirely from the onset of gastrulation. The notochord is seen to lie directly on top of the yolk extension. *cas* has been shown to encode a member of the Sox family of transcription factors and is thought to act cell autonomously.
Fig. 4.5. *p2x4 in situ* hybridisation staining on wild type (wt, A,B) and *bonnie and clyde* (bon, C,D,E,F) mutant embryos at 3 days. All panels show anterior to the left and dorsal to the top. All wt embryos show staining in the brain ("B"), notochord ("N") and gut (arrowheads). bon mutant embryos show staining in the brain ("B") and notochord ("N") as well as variable staining in the gut (arrowheads). For more complete description please see accompanying text.
Fig. 4.6. *p2x4* in situ hybridisation on wild type (wt, A, C) and *fau* (B, D) mutant embryos. All panels have anterior to the left and dorsal to the top. Staining is seen in the brain ("B"), notochord ("N") and gut (arrowheads). Mutant embryos show reduced gut staining. Staining in the bifid heart is probably trapping ("H"). For a more complete description of *p2x4* expression in these embryos see accompanying text.
downstream of both *bon* and *fau* (Kikuchi *et al.*, 2001, Alexander *et al.*, 1999). *p2x4* expression in *cas* is normal in the brain and notochord, where faint staining is detected just above the yolk extension (Fig. 4.7B and 4.7D). Expression in the gut is seen to be completely absent (Fig. 4.7B and 4.7D) when compared to wild-type siblings (Fig. 4.7A and 4.7C).

A combination of mutant and overexpression studies have been used to make a tentative hierarchy of genes involved in endoderm specification. *bon* and *fau* act both downstream of *nodal* and in parallel to each other. *cas* is expressed downstream of both *bon* and *fau*. *cas* then in turn activates endoderm specific genes such as *axial*, *fkd7* and *Sox17* (Reiter *et al.*, 2001).

### 4.6. Discussion

#### 4.6.1. Analysis of zebrafish p2x4

Further studies of the zebrafish P2X$_4$ receptor would benefit greatly from the production of an antibody against the protein. The zebrafish P2X$_4$ receptor was analysed by looking at the mRNA expression pattern during development and it is possible that discrepancies could occur between mRNA expression and protein localisation.

#### 4.6.2. Pharmacology of zebrafish p2x4

To date, the pharmacology of the zebrafish P2X$_4$ receptor has not been reported. The pharmacology of *p2x4* paralogues in rats and humans have been extensively studied (Khakh *et al.*, 1999, Miller *et al.*, 1998), and a lot of data has been published about the key amino acids controlling the binding affinity of agonists and antagonists (Clarke *et al.*, 2000, Carpenter *et al.*, 1999, Garcia-Guzman *et al.*, 1997, Bo *et al.*, 1995). Studies comparing the pharmacology of another zebrafish P2 receptor gene (*p2x3*) have shown that large differences occur between the fish gene and paralogues in other species. Pharmacological characterisation of the zebrafish P2X$_4$ receptor would be extremely interesting as it would allow us to find out if this unique pharmacological profile was
Fig. 4.7. *p2x4 in situ* hybridisation on wild type (wt, A,C) and *cas* mutant (cas, B,D) embryos. All panels show anterior to the left and dorsal to the top. Staining is seen in the brain ("B"), notochord ("N") and gut (arrowheads) of wt embryos. *cas* embryos show brain expression but lack all gut expression. The notochord is seen directly above the yolk extension. For a full description of the *cas* mutation see accompanying text.
4. Characterisation of a P2X$_4$ receptor in zebrafish

receptor subtype or species specific. It is possible that all the fish homologues of this receptor family may show fast channel kinetics.

4.6.3. Expression of p2x4 in zebrafish

Reports of the expression pattern of the P2X$_4$ receptor in other species have shown it to be expressed in a wide variety of tissues. The zebrafish p2x4 gene, however, appears to have a more restricted expression pattern. The gene is found to be present in the stomach and gut of larval and adult fish, from two and a half days of development onwards. This expression pattern of p2x4 is in agreement with studies showing P2X$_4$ receptor expression in the stomach, colon and bladder of rats (Yunaev et al., 2000, Tanaka et al., 1996, Bo et al., 1995). Frozen sections of zebrafish embryos reveal that the gene is also expressed at low levels in the brain, notochord, branchial arches and jaw.

Analysis of the expression of p2x4 in GIT mutants confirms that the expression is found in tissues that are endodermal derivatives. As the three mutants lack expression of the gene at all stages, p2x4 is probably expressed in tissues that are derived from both early and late developing endodermal tissues. Expression in bon but not cas, for instance, would show that the gene was only present in later deriving tissue.

The expression of p2x4 during zebrafish development is late, only appearing after the GIT has been formed. This suggests that the gene is not needed for the developmental control of endodermal tissues and cells that will form the gut. Instead, it probably acts as a housekeeping gene, needed for correct function of the gut after it has developed. This hypothesis is supported by the presence of p2x4 on adult tissue.

Expression in sections of one month old zebrafish larvae shows that by one month the expression has been lost from the retina and brain. It is still present in the GIT and notochord and a few cells are seen to express the gene.
in the branchial arches and jaw. It is not clear why the contents of the stomach also stain blue during the *in situ* hybridisation staining. One possibility is that gut wall cells have sloughed off and ended up in the lumen of the gut. This would account for some of the blue staining in the gut contents.

4.6.4. *Comparison to expression of P2X<sub>4</sub> in other species*

Similar to expression of the zebrafish *p2x3* gene (see chapter 3), *p2x4* is not expressed in as many different tissues as paralogous genes in other species. It is possible that following an extra genome duplication in the zebrafish (Postlethwait *et al.*, 1998) another orthologue of *p2x4* exists. There has been no evidence for this in the literature so far, and screening of the Sanger centre genome database has not detected any sequence that might be part of the orthologous gene. Alternatively, other P2 family members or ion channels may have been selected during evolution of the fish to undertake the function that *p2x4* fulfills in other species.

4.6.5. *Morpholino against p2x4*

In order to produce a functional fish *p2x4* knock-out it would be possible to design a morpholino against the first few amino acids of the sequence. Injection of the morpholino at single cell stage would block translation of the protein product and so remove receptor function. Morpholinos have been shown to be active for up to a week and so the late expression *p2x4* would still be inhibited following an injection at the single cell stage.

It would be difficult to assess the effectiveness of a *p2x4* knock-out without first designing a suitable test for fish GIT function. Without a P2X<sub>4</sub> receptor antibody it would not possible to show that the protein had been completely removed. During early development, before the GIT is formed, waste products are able to diffuse through the skin and pass into the water. After the GIT has formed, waste products are secreted as small hard pellets. Although some studies do exist, the fish has not been well characterised.
4. Characterisation of a P2X₄ receptor in zebrafish

behaviourally and it is hard to determine signs of discomfort that might ensue following a removal of normal GIT function. Suppression of expression in the brain and retina could be measured by devising a functional test against a brain lesion in that area.

4.6.6. Function of p2x4 in zebrafish

The most likely function of the P2X₄ channel during the life of the zebrafish larvae and adult is that it is acting as a post-synaptic ion channel in the enteric nervous system. P2 receptors have been described as being expressed in all hollow organs of animals. In a similar manner to the role of the P2X₃ receptor in the bladder, ATP could be released from the GIT on stretching of the gut epithelium. The ATP could act on P2X₄ channels to initiate peristalsis or excretion of waste products.

4.6.7. Future work

Future work on the zebrafish P2X₄ receptor gene could include making an antibody against the protein produce and library screening to search for another zebrafish p2x4 orthologue. Pharmacological analysis of the zebrafish receptor would also be extremely interesting. In order to do this it would probably be necessary to express the channel in a Xenopus oocyte and then use patch-clamp apparatus to assess pharmacological properties. Analysis of all these separate pieces of information may well suggest new therapeutic uses for agonists and antagonists of the P2X₄ receptor.
5. Embryonic expression of a P2Y₁₁ receptor in zebrafish

CHAPTER FIVE

Embryonic expression of a P2Y₁₁ receptor in zebrafish

5.1. Abstract

Studies in other species have shown that the P2Y₁₁ receptor is expressed in many human tissues. It is the only P2Y receptor to both activate two different signalling pathways and to form an intragenic splice variant with the SSF1 gene. The channel has been shown to have a role in the maturation of HL-60 cells to neutrophils and may be involved in the pathogenesis of cardiomyopathy or renal disease following the cytolytic release of ATP from damaged tissue. However, little is known about the expression and function of the P2Y₁₁ receptor during embryonic development.

This chapter describes the acquisition and expression of a novel zebrafish P2Y₁₁ receptor during development. Possible functions for the receptor are also discussed. This is the first description of any P2Y receptor in the zebrafish during development. The receptor is first expressed during gastrulation and expression continues well into larval development.

5.2. Introduction

The P2Y₁₁ receptor has only been relatively recently discovered and thus far only the human receptor has been described. The P2Y₁₁ receptor is a seven transmembrane domain G protein coupled receptor. It has been shown to be a selective purinoceptor that is potently inhibited by antagonists. Human P2Y₁₁ was cloned from a placenta library by low homology screening (Communi et al., 1997) and has shown to be expressed in foetal human brain, kidney, muscle, testis, liver and spleen as well as in the small intestine and cells of the immune system (van der Weyden et al., 2000, Communi et al., 1997). The P2Y₁₁ receptor is unique among P2 receptors in that it is able to both activate two
5. Embryonic expression of a \( \text{P}_2\text{Y}_{11} \) receptor in zebrafish

different signalling pathways and to undergo intragenic splicing with an \( \text{SSF1} \) gene. Activation of the receptor causes an increase in the levels of intracellular calcium ions, formation of cAMP and activation of PKC.

For a more complete description of the pharmacology, tissue distribution and function of \( \text{P}_2\text{Y}_{11} \) receptors in other species, please refer to the general introduction in chapter 1 of this thesis.

5.3. Pharmacological properties

\( \text{P}_2\text{Y}_{11} \) is the only P2Y receptor found so far that selectively uses ATP as its naturally occurring agonist (von Kügelgen and Wetter, 2000). It is also potently activated by ATP analogues including ATP\( \gamma \)S and 2-propylthio-\( \beta,\gamma \)-dichloromethylene-d-ATP (Communi et al., 1999). Suramin acts as a strong inhibitor of the receptor and reactive blue 2 acts as a less potent inhibitor. The receptor is only slowly desensitised and recovers quickly following the removal of the agonist (Suh et al., 2000).

The \( \text{P}_2\text{Y}_{11} \) receptor is unusual in that it is able to signal by two separate pathways upon activation by an adenine nucleotide. Studies in a large number of cell lines, including human promyelocytic leukaemia cells (HL-60, Qi et al., 2001; Jiang et al., 1997), Madin Darby canine kidney cells (MDCK, Zambon et al., 2001) and pancreatic duct endothelial cells (PDEC, Nguyen et al., 2001) have shown that the \( \text{P}_2\text{Y}_{11} \) receptor is able to activate both the standard phospholipase C (PLC) pathway as well as the enzyme adenylate cyclase. There are conflicting reports as to whether both pathways are activated with the same efficiency, but studies by Qi et al (2001) suggest that adenylate cyclase is activated 15 times less efficiently than PLC.

5.4. Molecular properties

The \( \text{P}_2\text{Y}_{11} \) gene is also interesting at the molecular level. It is the only \( \text{P}_2\text{Y} \) gene to have been found so far that has two introns (Communi et al.,}
5. Embryonic expression of a P2Y<sub>11</sub> receptor in zebrafish

The first intron contains the initiation methionine and a phosphorylation site. The second intron contains sequence for all seven transmembrane domains.

P2Y<sub>11</sub> can form intragenic splices with another human gene called SSF1 (Communi et al., 2001). SSF1 is a homologue of the gene supressor of swi four in yeast and is related to the Drosophila gene peter pan (ppan, Migeon et al., 1999). The yeast homologue of ppan has a function in controlling mating between yeast cells. ppan has a similar function in Drosophila development; it controls larval growth. Intragenic splicing occurs by the loss of the first intron and the addition of an ATP binding site to the P2Y<sub>11</sub> gene following SSF1 binding (Communi et al., 2001). The function of this splice formation is not yet understood, particularly since the pharmacological properties of the receptor do not appear to have changed when measured in vitro. It is possible that in vivo the pharmacological profile would be different, or that the splice has a function that has not yet been discovered.

5.5. Tissue distribution and function

mRNA analysis of the human P2Y<sub>11</sub> receptor has shown it to be expressed in human foetal brain, kidney, testis, muscle, liver and spleen (van der Weyden et al., 2000). In HL-60 cells it has shown to be involved in the maturation into granulocytes (Jiang et al., 1997) and in PDEC it has been shown to act to increase the secretion of fluids and electrolytes into the pancreatic duct (Nguyen et al., 2001). In the immune system, P2Y<sub>11</sub> receptors mediate the maturation of dendritic cells following ATP stimulation (Wilkin et al., 2001).

5.6. Results

An EST clone that showed high homology to the human P2Y<sub>11</sub> receptor when compared using a BLAST (Basic Local Alignment Search Tool, www.ncbi.nlm.nih.gov) was obtained from the Washington University EST database (www.wuzgr.edu). The EST clone was a fragment of around 600
5. Embryonic expression of a P2Y_{11} receptor in zebrafish

base-pairs in length and did not contain a full length sequence. The sequence has not yet been published and so there is no corresponding GenBank accession number. The fragment was long enough to make an in situ hybridisation probe. In order to obtain the full length sequence of the gene, the Sanger centre database (www.sanger.ac.uk) was screened using a trace repository search. The search identified several fragments that showed high homology to the EST clone. Using a contig assembly program ("CAP", http://bioweb.pasteur.fr/seqanal/interfaces) a contig was assembled that appeared to encode the full length reading frame of the P2Y_{11} receptor. While it is impossible to verify this without cloning and sequencing the full length receptor from a zebrafish library, the contig started at an initiation codon, ended in a stop codon and was of a similar base-pair length to the closest related human homologue. The p2y11 gene constructed using this method has an open reading frame of 915 base-pairs and codes for a protein that is 305 amino acids long (Fig. 5.1.)

5.6.1. Tree diagram to compare homology to other species

A homology tree comparing the amino acid sequence of every P2Y receptor in different species was constructed (Fig. 5.1.). Several other P2Y receptor orphans were included. The tree was based on a clustal W alignment and was made using the treepuzzle program (Treepuzzle 5.0, http://www.nsc.liu.se/software/biology/puzzle5/). The zebrafish clone (Fig. 5.1., outlined in black) showed the highest homology to both the human P2Y_{11} and the frog P2Y_{1} receptor. Genbank accession numbers for all the genes are included at the bottom of figure 5.1. The accession numbers for the zebrafish P2Y_{11} receptor and the human P2Y_{13} receptor are not yet available.

A Jotun Hein alignment (Hein, 2001) of zebrafish p2y11, human p2y11 and *Xenopus* p2y1 was constructed using the Lasergene 5 analysis program (DNASTar, Fig. 5.2.). The *Xenopus* p2y1 gene was included in the alignment as it showed high homology to the zebrafish and human p2y11 genes in a
Figure 5.1. Homology comparing sequences of P2Y receptors in a wide variety of species. Sequences were aligned using a Clustal W program and the tree was made using the Treeview software. See results section for a more complete description and discussion.

Genbank accession numbers: Chick p2y1, X73268; Mouse p2y1, AJ245636; Human p2y1, XM_003035; Rat p2y2, U56839; Rat p2y4, Y14705; Human p2y4, X91852; Human p2y11, XM_053314; Frog p2y1, AF432354; Human GPR17, XM_002705; Human p2y6, AF007892; Mouse p2y6, AF298899; Turkey p2y3, AF069555; Chick p2y3, X98283; Human p2y10, AF000545; Human p2y9, U90322; Human p2y5, AF005419; Human p2y12, XM042202; Human GPR87, XM042204; Rat p2y12, AF313450; Frog p2y8, X99953. Accession numbers for zebrafish p2y11 and Human p2y13 are not yet available.
5. Embryonic expression of a P2Y\(_{11}\) receptor in zebrafish

Figure 5.2.

Jotun Hein alignment of zebrafish \(p2y11\), human \(p2y11\) and frog \(p2y1\). The zebrafish gene is unpublished, the sequence was obtained from the Sanger centre database. The genes show low homology to each other. The human and zebrafish homologues have about 20\% homology. Comparison of the sequences suggests that the Xenopus \(p2y1\) gene actually codes for a P2Y\(_{11}\) receptor. For genbank accession numbers please refer to the legend for figure 5.2.
Figure 5.2.

```
        Majority
        Zfish p2y1l
        Human p2y1l
        Frog p2y1
        
        10  20  30
        M X ---------- A X X S X A S ------
        
        1
        M X ---------- ANVSGAKS
        
        1
        M X ---------- NDSLCKES
        
        1
        M X ---------- G F Q X D X L P P X Y

        Majority
        Zfish p2y1l
        Human p2y1l
        Frog p2y1
        
        40  50  60
        V F S X N L A X S D X L Y X L T L P X L I X Y X X K K X W

        Majority
        Zfish p2y1l
        Human p2y1l
        Frog p2y1
        
        100 110 120
        X F G D A X C K L E R F L F T C N L Y G S I X F I T C I S V

        Majority
        Zfish p2y1l
        Human p2y1l
        Frog p2y1
        
        244 254 264
        N R Y X G I V H P F F X R X S L R P K H A X X X S A L V W F

        Majority
        Zfish p2y1l
        Human p2y1l
        Frog p2y1
        
        160 170 180
        I V I X X X S P X L S F S G T X X X X X X X G X ------

        Majority
        Zfish p2y1l
        Human p2y1l
        Frog p2y1
        
        190 200 210
        I V I V I X X S P X L S F S G T X X X X X X X G X ------

        Majority
        Zfish p2y1l
        Human p2y1l
        Frog p2y1
        
        220 230 240
        X X C I X C X D T S X X X X L X S Y X X Y S L X L X V X G C
```
Fig. 5.2. Jotun Hein alignment of zebrafish p2y11, human p2y11 and Frog p2y1. The different receptors show about 30% homology to each other. The high degree of homology between frog p2y1 and the other p2y11 clones suggests that it may actually be a p2y11 receptor. For GenBank accession numbers, please refer to figure 5.2.
5. Embryonic expression of a P2Y₁₁ receptor in zebrafish

homology tree (Fig. 5.1.). Comparison of the sequences shows that the zebrafish homologue has very low sequence identity to the other genes. The human and zebrafish homologues show 30% identity at the amino acid level. The low degree of homology made it difficult to predict the position of the seven transmembrane domains or of key amino acids shown to be important for ligand binding and phosphorylation of the human receptor (see Communi et al., 1997).

5.6.2. In situ hybridisation expression pattern

In order to study the expression of the zebrafish P2Y₁₁ receptor during development, an in situ hybridisation probe was transcribed from the EST fragment. The mRNA coding for the receptor was detected from the middle of gastrulation onwards. At 50% epiboly expression was seen in cells of the gastrulating embryo immediately adjacent to the yolk cell. The cells were present in a uniform stripe above the yolk cell but were never seen in the dorsal most cells of the embryo (Fig. 5.3A). This expression pattern is essentially conserved throughout all the developmental stages studied. At 70% epiboly, tailbud, early somitogenesis stages through to 18s somites (Fig. 5.3B-5.3H) expression is seen in cells next to the yolk cell but never in the more dorsal cells of the embryo. During some of the early gastrulation stages (Fig 5.3B and 5.3C) the expression appears to be reduced at either side of the embryos (including the area at which the shield develops). This may be due to the position of the embryo when photographed. At later stages (5.3D-5.3H) the staining is seen in a clear anterior to posterior stripe. This expression may correlate with tissue that has been specified as forming mesendodermal derived tissues.

Later on in development the expression of p2y11 is seen to be well conserved. At 24 hours (Fig. 5.4A and 5.4C) expression is seen in the anterior tissue of the embryo. Strong expression is seen in the head and is present in tissue above the yolk cell. However, no expression is detected in the tail. A higher magnification view of the embryo (Fig. 5.4C) shows that the expression is
Fig. 5.3. *In situ* hybridisation staining of *p2y11* on zebrafish embryos. Panels A, B, C, D, E, G and H all show lateral views with anterior to the left and dorsal to the top. Panel F is an anterior view with dorsal to the top. From gastrulation onwards staining is seen in a tissue layer that lies above the yolk cell. It is possible that *p2y11* is present in all mesendodermal cells. Later stages are shown in the next figure. For a more complete description of the staining please refer to the text in the results section.
Fig. 5.4. *In situ* hybridisation of *p2y11* on 24 hour (A, C) and 48 hour (B, D) embryos. Staining is seen in a tissue layer which lies dorsal to the yolk and yolk extension. Staining is seen to be stronger in anterior tissue and is very weak or not present in tail tissue. *p2y11* may be present in mesoderm and endoderm derived tissue. All panels show anterior to the left and dorsal to the top.
5. Embryonic expression of a \( P2Y_{11} \) receptor in zebrafish

not seen in the dorsal-most or anterior-most tissue of the embryo. \( p2y11 \) expression is seen to be the same at 48 hours as was described for 24 hour embryos (Fig. 5.4B and 5.4D). The expression is restricted to the head of the embryo. Expression is seen in cells that lie adjacent to the yolk cell; the tail shows no expression.

5.7. Discussion

This chapter presents the first evidence for a P2Y receptor active during zebrafish development. Expression of the gene was seen from the middle of gastrulation onwards, and expression was detected until much later in development.

5.7.1. Tree diagram of sequence homology

Construction of a homology tree containing all P2Y genes has given some interesting insights into the sequence homology of previously described receptors. P2Y receptor orphans were included as well as named P2Y receptors to assess their homology to known P2Y family genes. The frog P2Y\(_1\) receptor was seen to have high homology to the human and zebrafish P2Y\(_{11}\) receptors. This is surprising and may suggest that the frog clone is not a P2Y\(_1\) receptor. The zebrafish P2Y\(_{11}\) homologue was initially described as a P2Y\(_1\) receptor on the Washington EST database and was only re-named P2Y\(_{11}\) after more detailed sequence analysis. It is possible that frog P2Y\(_1\) was named and described before the P2Y\(_{11}\) receptor family was discovered.

The P2Y\(_2\) and P2Y\(_4\) subfamily of receptors show high homology to each other and may in actuality be part of the same subfamily. Rat P2Y\(_2\) in particular is closely related to P2Y\(_4\) receptors. Two P2Y family orphans have been included in the homology tree. These are called human GPR17 (G protein coupled receptor 17) and human GPR87. Comparison of these sequence in a tree diagram suggests that they may indeed be part of the P2Y family of receptors. Human GPR17 appears to be related to the P2Y\(_3\) and P2Y\(_6\) receptor...
subfamily. Human GPR87 on the other hand appears to be closely related to the rat P2Y12 receptor. There is already a human homologue named P2Y12 and so it is not clear how GPR87 will fit into the P2Y family. Human GPR87 is also closely related to the rat P2Y12 receptor, suggesting that it may not simply be a splice variant of the human paralogue. It will be extremely interesting to see how future analysis and research places these and other new P2Y genes into the P2 receptor family.

5.7.2. Sequence alignment

Sequence alignment of the zebrafish P2Y11 receptor, the human P2Y11 receptor and the frog P2Y1 receptor confirms that the zebrafish paralogue has very low homology to its human counterpart. This low homology makes it very difficult to deduce the position of the seven transmembrane domains and other conserved amino acids. The low homology is not surprising, as comparison of the human clone to its nearest related channel (the P2Y1 receptor) also shows very low homology. Zebrafish homologues are often seen to be both highly divergent from other mammalian paralogues and closely related to frog genes (see tree diagram, chapter 4, figure 4.2). This closer relationship is in agreement with the results of the homology tree.

Another possibility for the high divergence seen between the human and zebrafish sequences is that the zebrafish base pair data that I extracted and constructed from the Sanger centre genome database is not correct. The only way to confirm or disprove this possibility would be to clone the whole gene from a zebrafish and sequence it. The gene was not found during several attempts to screen zebrafish libraries at different developmental stages. In theory isolating the sequence from the Sanger database should work in a similar way to a traditional library screen. In both cases the clone is found by sequence homology and therefore the clone that I have assembled should be recognised by the same homology when screening a zebrafish library. The clone starts at an initiation codon and ends at a stop codon. It codes for an protein of the
length that would be expected from studies in other species (see Communi et al., 1997).

5.7.3. p2y11 in situ hybridisation expression

The expression pattern of p2y11 during zebrafish development is extremely interesting and does not resemble the expression pattern of any other gene during development described thus far. It is likely that the receptor is expressed in mesendodermal tissue before the two germ layers have divided into separate entities. In later developmental stages, as the expression pattern is essentially the same as that seen in gastrulating embryos, the receptor may be expressed in both mesodermal and endodermal tissue. This is the earliest reported expression of any P2 receptor during development so far and is the only description of a P2Y receptor active during zebrafish development.

5.7.4. Function of the P2Y11 receptor during development

Such a novel expression pattern of a gene during development makes it difficult to ascribe possible functions to the zebrafish P2Y11 receptor. There is no data available concerning the role of p2y11 in the development of species with which the zebrafish paralogue can be compared. The expression is first detected at a very early stage of development. Information from studies in a variety of cell lines, including HL-60 cells (Jiang et al., 1997), suggest that the receptor may be involved in controlling the maturation or differentiation of cells. This may be one possible function of the receptor in the mesendodermal cell layer. Another possibility, suggested by the ability of human p2y11 to increase the activation of renin (van der Weyden et al., 2000) is that the receptor is involved in the activation of downstream target genes that are necessary for normal development of the mesendodermal cell layer.

In order to address this question a morpholino against the p2y11 gene may be of interest. Once again, it is important to design a functional test that would allow the efficacy of the morpholino to be tested. If p2y11 is indeed
5. Embryonic expression of a P2Y_{11} receptor in zebrafish

important for the transcription of genes during early development then knocking out the function of the gene may result in failure of the embryo to gastrulate properly. This is an interesting phenotype in itself but it would then be difficult to further elucidate the underlying mechanisms causing failure of gastrulation. Failure of gastrulation is often seen following injection of high concentrations of morpholinos or RNA, making it hard to judge whether or not this is a specific phenotype. The expression of P2Y_{11} in zebrafish is the earliest expression of any P2 receptor during development and may well be of great interest in the study of P2 gene function during development.

5.7.5. Future plans

This chapter provides the first description of a P2Y gene during zebrafish development. The expression pattern of the gene coding for the receptor has a unique and unusual pattern.

All the results and conclusions about the expression and function of this gene are based on in situ hybridisation analysis of the mRNA coding for the receptor. An antibody against the P2Y_{11} protein would be useful to examine the expression of the protein product. In light of the unusual mRNA expression this could be extremely interesting. The expression pattern appears to demarcate a boundary between different developmental cell types, although no boundaries have yet been described in this position in the fish embryo.

A description of the role of P2Y_{11} receptors during the development of other species would also be useful and interesting. One of the main objectives and strengths of using the zebrafish to study the function of P2 receptors during development has been to see how P2 receptor expression and function has been conserved during evolution. It would be interesting to see if this mesendodermal expression pattern was present in other species. Genome duplication in the fish also raises the possibility that another zebrafish P2Y_{11} receptor orthologue exists (Postlethwait et al., 1998, Holland et al., 1996).
5. Embryonic expression of a P2Y₁₁ receptor in zebrafish

Analysis of the expression in other species may well show that the receptor is expressed in an increased number of places than the zebrafish paralogue. This has been verified by studies of the fish P2X₃ and P2X₄ receptors (see chapter 3 and chapter 4 of this thesis).

Another possibility for future experiments would be to analyse the promoter of p2y11 and to search for genes that are activated upstream. A library screen for other P2Y receptors that are active in zebrafish development could also be very useful. If other P2Y genes are active so early on in development, then the whole subfamily may prove to be extremely important in the early development of the embryo. Neither the Sanger centre database or the Washington EST database have yet produced fragments of other P2Y receptors. Genome sequencing will be completed shortly and it will be then possible to both find all related orthologues and to find exact numbers for P2Y receptors active during fish development.
6. Screening for novel zebrafish mutants

CHAPTER SIX

Screening for novel zebrafish mutants

6.1. Abstract

In two separate genetic screens, novel zebrafish mutants were identified and characterised in order to further understand the process of axon scaffold formation during zebrafish development. The first screen, carried out at University College London, used a cocktail of in situ hybridisation probes to look at the formation of the anterior neural plate as well as looking at 24 hour morphology and anti-acetylated tubulin antibody staining at 48 hours. The second screen, carried out at the Max Planck Institute for Developmental Biology, Tübingen, used both 24 hour morphology and 32 hour anti-acetylated tubulin staining to identify embryos with axon pathfinding defects. In both screens, the mutants were re-identified and further characterised. The introduction to this chapter describes the history of screening and the design of these two screens. The results of both screens are presented and discussed.

6.2. Introduction

The function of a gene of interest can be best understood by looking at information from a wide variety of sources. These include looking at gene product and protein distribution, mutations, injection of anti-sense RNA, morpholinos or other approaches to analyse loss of function phenotypes (Haffter et al., 1996). The gene and protein distribution can be detected by in situ hybridisations and antibody stainings respectively. A combination of these approaches will often be employed to elucidate the function of the gene of interest.

The best studied developmental model organism is the fruitfly, Drosophila melanogaster, in which a very powerful combination of genetics
and molecular biology has allowed many developmentally important genes to be found. The majority of the genes that control patterning along the anterior-posterior and the dorsal-ventral axes have been identified by large scale saturation mutagenesis screens (Nüsslein-Volhard and Wieschaus, 1980, Nüsslein-Volhard et al., 1985, Wieschaus et al., 1984). Further genes have also been found by more sophisticated mutagenesis screens, as well as by using molecular biological and biochemical methods.

6.2.1. Conservation of genes between species

The large degree of conservation of genes between different organisms during evolution has subsequently allowed many of the homologues of invertebrate genes to be found in vertebrates. In many cases, these play similarly crucial roles during development. As a result of this conservation, developmental studies in one species can give insights into the development of other unrelated species.

6.2.2. Gene duplication and redundancy in zebrafish

Gene duplication in the zebrafish during evolution has increased the complexity of the genome. Zebrafish are thought to have undergone an additional duplication event as compared to mammals and have multiple copies of genes that are found in vertebrate ancestors (Van de Peer, 2001, Postlethwait et al., 1998, Holland et al., 1996). An interesting result of this high conservation of gene families across species is shown in the redundancy of genes that have multiple homologues in some species compared to others. An example of redundancy in gene function is seen in the Hedgehog family of genes. Hedgehog was first discovered in Drosophila melanogastor (Nüsslein-Volhard and Wieschaus, 1980) and subsequently homologues have been discovered in other species. Both rodents and zebrafish are known to have three Hedgehog homologues-known as sonic, indian and desert hedgehog in rodents (Echelard et al., 1993) and sonic, echidna and tiggywinkle hedgehog in zebrafish (Krauss et al., 1993). The three homologues have overlapping domains of expression and appear to
6. Screening for novel zebrafish mutants

also have overlapping functions that mirrors those of the Drosophila genes. Screening for novel developmental phenotypes and the cloning of the genes in one organism therefore allows developmental pathways to be deduced in a variety of species.

6.3. Mutagenesis screens

Large scale mutagenesis screens provide a good way of attempting to systematically search for these essential pattern forming genes. Not all genes will be found by a mutagenesis approach, only those that show a non-redundant, easily visible phenotype in the organism of choice. Molecular pathways leading to the development of a certain feature or organ may be defined by groups of mutants that show similar phenotypes. By repeated rounds of screening it is theoretically possible to reach near saturation for these genes, so that in theory all the genes involved in a developmental process have been found. Redundant genes, or those with a less obvious visible phenotype will be difficult to recover from a large scale mutagenesis screens.

6.3.1. Organisms used in screening

Mutagenesis screens have been carried out in several model organisms, including Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana, mouse and zebrafish. The choice of the model organism to be used in screening has to take into account the ease of identification of mutant phenotypes and the ability to clone genes after mutagenesis (Nüsslein-Volhard., 1994). The fish is an ideal vertebrate model to screen for interesting and highly specific phenotypes, as the embryos are completely transparent and develop rapidly. The generation time is short and large clutches of embryos are produced, often in excess of 100 from a healthy adult. By 24 hours it is possible to see most of the organs that will be present in the adult. Free swimming larvae at 7 days exhibit many behavioural and morphological traits of the parent but are only a few millimetres long (Kimmel, 1989).
6. Screening for novel zebrafish mutants

6.3.2. Mutagenic agents

There are a variety of mutagenic agents that have been used to generate fish to be screened for novel phenotypes. The two main points that have to be taken into account when choosing a mutagen are the efficiency and the ability to make specific mutations. Although X rays, transposons and gamma radiation have all been used for this purpose (Kimmel, 1989), by far the most favoured agents in zebrafish are the chemical mutagens, EMS (Ethylmethanesulphonate) and ENU (N-ethyl-N-nitrosourea). Both EMS and ENU frequently produce intragenic point mutations making them ideal for the setting up of a genetic screen. The frequency of mutation can be tested by looking at mutations in known pigment loci, or by measuring the number of embryos with an embryonic lethal phenotype. An average mutation frequency in a screen is usually around 1 mutation in a defined pigmentation locus per every 700 – 800 genomes screened (Solnica-Krezel et al., 1994). Although ENU causes more death to fish during the treatment regime, it has a higher mutation frequency and is now the preferred mutagen in genetic screens. The adult males that will be used to generate mutant progeny are immersed in a solution of ENU that ranges between 2.0 to 3.5mM for one hour a week. After four weeks of treatment the fish are left to recover before the start of the screen. One of the main problems to be addressed when setting up a screen is that of mosaicism in the spermatozoa of the male fish. To avoid this possibility, the germ cells in the male fish are mutagenised at a premeiotic stage during spermatogenesis. A point mutation induced in one DNA strand of a premeiotic germ cell will be fixed in both strands during DNA replication. In the three or four weeks following the ENU treatment regime the mutagenised male fish are mated to wild-type female fish and the progeny from these crosses are discarded. After one month of this breeding regime, the sperm that was present in the spermatogonia during mutagenesis should have been removed. The fish will then be able to produce new spermatozoa carrying non-mosaic copies of the mutation of interest. (Solnica-Krezel et al., 1994, Mullins et al., 1994). ENU has an active O\(^6\) – ethylguanine group that
6. Screening for novel zebrafish mutants

causes alkylation of one strand of DNA in the gene of interest (Mullins et al., 1994). The major change that occurs as a result of this is thought to be a shift from a GC pair to an AT pair in the DNA code. Comparing the sequence of genes of interest from mutant fish compared to wild-type has also confirmed this shift of base pair.

Several successful screens have been carried out in zebrafish, mouse and C.elegans using proviral insertions. In both mouse and zebrafish, mouse retroviruses have been successfully used to generate novel insertion lines (Amsterdam et al., 1999). Although the efficiency of mutation in an insertional mutagenesis screen is around 9 times lower than that seen in a ENU mutagenesis screen, the mutated genes are easily identified as the proviral insert provides a sort of genetic tag (Amsterdam et al., 1999).

6.3.3. Haploid screening

There are two types of mutagenesis screen that can be undertaken using the zebrafish: haploid or diploid screens. Haploid screens are less labour intensive and need less aquarium space, whereas diploid screens allow for the better recovery of mutations after initial identification. Diploid screens provide more consistent and reliable results and so are favoured by most research laboratories.

Haploid embryos can be produced gynogenetically or androgenetically (Westerfield, 1995, Corley-Smith et al., 1996). In gynogenetic haploid screens, mutagenised females are squeezed to produce a clutch of unfertilised embryos. The embryos are kept away from excess water to stop the chorion from swelling and therefore preventing fertilisation. Sperm to be used in the fertilisation is treated with a pulse of UV irradiation, which cross the links the DNA (and therefore deactivates the nucleus) but does not affect its ability to activate the egg. Fertilisation is performed in vitro by careful mixing of the sperm and eggs and the embryos develop with only a maternal contribution to the genome (Westerfield, 1995). Conversely, in androgenetic screens, the
6. Screening for novel zebrafish mutants

Unfertilised eggs are UV irradiated and are subsequently fertilised with untreated sperm from mutagenised males. This in turn produces haploid embryos with only a paternal contribution to the genome (Corley-Smith et al., 1996).

6.3.4. Diploid screening

Haploid screens produce embryos that contain a background phenotype which itself can make the scoring of novel phenotypes difficult. The embryos have a reduced body length, a kinked neural tube and smaller melanocytes than diploid wild type embryos (Streisinger et al., 1981). Although present, these differences are consistent enough to be discounted in the screening process making the finding of new mutations possible. An alternative to screening haploid progeny is to screen diploid mutants.

6.3.5. Gynogenetic diploid screening

Diploid screens require a lot more aquarium space and time to produce new mutants, though the mutations are easier to spot and recover than those found by haploid screening. In gynogenetic diploid screens, eggs from a mutagenised mother are fertilised with sperm that has been UV treated (Streisinger et al., 1981). The union of sperm and egg serves two main functions - to initiate cleavage in the egg (initiation) and to restore a diploid set of heterozygous chromosomes. UV treatment of sperm destroys the DNA in the nucleus, but still allows initiation to occur. The egg is then subjected to either heat shock or to early or late hydrostatic pressure to restore a full complement of chromosomes. By either suppressing meiosis II (early pressure) or the first cell cleavage (late pressure) the maternal chromosomes are duplicated and a homozygous diploid embryo is produced (Streisinger et al., 1981). In theory, as there is no sexual contribution to this process, all the embryos produced in a clutch will be clones of each other, although recombination in the germ line will produce some differences between embryos.
6. Screening for novel zebrafish mutants

6.3.6. Classical three-generation screening

The first Tübingen screen, which ran from 1993 until 1996, was a good example of a classical three generation diploid screen in zebrafish. A large scale morphology screen was undertaken to identify genes in the fish with unique and essential functions. The mutants that were identified were divided into over 20 different groups depending on morphological similarities (see Development Vol. 123, 1996). The groups that mutants were assigned to were preliminary, with little characterisation of the mutations having been done at this point in time. The mutants were identified by screening for gross morphological defects. No antibodies or \textit{in situ} hybridisation probes were used to identify new mutants.

6.3.7. Limitations of genetic screens

Screening for mutants that only affect morphology will severely limit the number of developmentally important genes that are isolated. Redundancy of genes during evolution means that some phenotypes will not be readily visible, even when the gene usually needed for normal development is mutated. Other limiting factors affecting genetic screens include a restriction in the size of aquarium available for crossing and keeping mutant lines, the number of fish screened and the number of embryos produced in every clutch by mated fish.

6.3.8. Tübingen screen 2000

In order to find further genes that have roles in specific developmental processes, a second large scale mutagenesis screen was designed. The screen ran from March 2000 until March 2001 and was conducted in the lab of Christiane Nüsslein-Volhard in the Max Planck Institute for Developmental Biology, Tübingen. Phenotypes were scored using antibodies, \textit{in situ} hybridisation probes and visible morphology where necessary, to target specific organ systems. The mutation groups that were screened for included defects in pigmentation, somitogenesis, fin development, eye morphology, brain axon guidance, blood formation, cartilage formation, neural crest
6. Screening for novel zebrafish mutants

formation and migration, otolith development, muscle development and vascular system development. This chapter describes the protocol and results for the brain screen.

6.4. Patterning of the zebrafish brain

The formation of axon tracts in the developing brain has been studied in a variety of organisms, both invertebrate and vertebrate (see Herrick, 1937 and Harrelson and Goodman, 1988). The developing fish brain is a good system to look at this problem because of the rapid development and transparency of the tissue. The tracts that are formed during the first few days of development are now well characterised and described (Chitnis and Kuwada, 1990, Wilson et al., 1990).

At 11 hours the brain shows few overt signs of patterning. However, a few hours later, neuromeres are visible in the embryonic brain anlage and by 18 hours ten main divisions of the brain can be seen. These include a telencephalon, diencephalon and midbrain as well as seven rhombomeres (Hanneman et al., 1988). These simple divisions do not persist for long and after one day of development further distinct divisions are visible including the epiphysis and cerebellum.

6.4.1. Genetic pre-patterning

One of the most exciting discoveries concerning the patterning of the brain was the finding that gene expression patterns divide the neural tube up into distinct areas. Genes that are important in this process in the zebrafish include krox 20 (Hindbrain, Wilkinson et al., 1989), pax 2.1a (Krauss et al., 1991), engrailed (Hatta et al., 1991), emx (Morita et al., 1995) and dix (Akimenko et al., 1994). These genes are expressed in spatially restricted patterns that are slightly different to the early morphologically visible patterning of the brain. The genes are forming a "prepattern" of the brain that will eventually lead to the formation of a correctly orientated axon scaffold.
6. Screening for novel zebrafish mutants

6.4.2. Genetic prepatternning in the MHB

A good example of pre-patterning in the fish is seen in the development of the midbrain-hindbrain boundary (MHB). The midbrain of the zebrafish includes the tectum, ventral tegmentum and the ventrolateral torus semicircularis. It acts as a relay station for sensory information from the eyes, ears and lateral line. The midbrain also contains both the trochlear and oculomotor cranial motor nuclei which are involved in the control of eye movements. During development, both the midbrain and the anterior hindbrain are patterned by an organising centre called the MHB. The area of brain that will become the midbrain is first specified during gastrulation, but it is not until the somitogenesis that the tissue in the midbrain and isthmus region folds up to form the MHB. The development of the MHB can be divided up into two separate steps. Early embryonic signals position the fold and establish the MHB. Later embryonic signals have a role in maintenance of the MHB and in controlling the expression of signalling molecules that are secreted to allow the MHB to function as an organiser.

In a similar way to the formation of the organising centre in the fly wing, the MHB is formed at the interface of expression of two genes: ot\(_x\)2 and gb\(_x\)1 (called Gbx2 in mice, Simeone et al., 1998, Wassarman et al., 1997). Studies in mouse have shown that disruption of the expression domain of one of these genes allows both an expansion of the expression domain of the other gene and a change in the position of formation of the MHB (Acampora et al., 1997). Each gene appears to act by inhibiting the expression of the other.

At the end of gastrulation, genes such as pax\(_{2.1}\), her\(_5\) and wnt\(_1\) (Miller et al., 1996, Krauss et al., 1992, Krauss et al., 1991) are seen as a chevron shaped area of expression in the midbrain tissue. The adjacent hindbrain tissue is seen to express fgf8. It is still not known which genes act upstream of ot\(_x\)2 and gb\(_x\)2, although a good candidate for this would be wnt\(_8\) (Erter et al., 2001, Lekven et al., 2001). Signals from the germ ring, including wnt\(_8\), are
thought to act in a concentration dependent manner to specify the genes activated in any one area.

Following positioning at the interface of *otx2* and *gbx2*, the MHB acts to pattern the anterior-posterior axis of the surrounding tissue. Signalling molecules including *fgf8*, *wnt1* and *pax2.1* are secreted from the MHB. *pax2.1* acts to initiate the expression of both *eng2* and *eng3*. Wnt1 protein functions as a mitogen and maintains expression of engrailed proteins, thereby patterning the surrounding neural tissue. Fgf8 protein is thought to both maintain gene expression in the MHB and to pattern the anterior hindbrain. Fgf8 inhibits the expression of *hox* genes in rhombomere 1 of the developing hindbrain, (Irving and Mason, 1999). *fgf8* secreted from the MHB may therefore control the anterior limit of *hox* gene expression during development.

The interaction and regulation of these three genes is complicated and becomes mutually dependent as development proceeds. *fgf8* is thought to be controlled by a feedback loop that involves other genes such as *sprouty4*, *fgf3* (Fürthauer et al., 2001). Further evidence for this feedback has come from the zebrafish mutant *aussicht* (Heisenberg et al., 1999).

Studies of the genetic pathways involved in MHB patterning in zebrafish have been helped by the characterisation of several mutants. *acerebellar* (*ace*) has a mutation in *fgf8* (Shanmugalingam et al., 2000) and *ace* embryos show a lack of MHB and cerebellum, with accompanied disturbance in the anterior-posterior patterning of the midbrain and in projection of retinal ganglion cells out of the midbrain (Picker et al., 1999). Expression of EphA2 and EphA5 is usually seen in a graded pattern in the hindbrain. In *ace* mutants, this gradient of expression is levelled and this may account for the mis-patterning of the anterior-posterior tectal axis (Picker et al., 1999). *no isthmus* (*noi*) mutants have a null mutation in *pax2.1* and lack midbrain, MHB and cerebellum. Mutant analysis has shown that *eng2* and *eng3* are strongly
6. Screening for novel zebrafish mutants

dependent on noi, whereas expression of fgf8 and wnt1 were unaffected in noi mutants. There are other zebrafish MHB mutants that show defects in midbrain patterning. ausicht mutants show upregulation of both fgf8 and pax2.1 (Heisenberg et al., 1999) and spiel-ohne-grenzen/pou2 mutants lack MHB and cerebellum in a phenotype that is somewhat similar to ace (Schier et al., 1996).

6.4.3. Patterning of the forebrain

Although the forebrain does not overlie a structure analogous to the floorplate, it is patterned by a combination of the Nodal, Hedgehog and Fgf pathways in a similar manner to more posterior brain structures. A lot of the information on this process comes from mutants in these pathways, where disrupted forebrain patterning is seen. During early brain morphogenesis, the mesendodermal tissue of the prechordal plate migrates underneath the developing forebrain. Together with the midline tissue of the anterior neuroectoderm the prechordal plate provides inductive signals for hypothalamus formation and the splitting of the eye field anlage (Kiecker and Niehrs, 2001). The most ventral tissue of the forebrain is the hypothalamus. From studies using the hypothalamic marker gene nk2.1a and the telencephalic marker gene nk2.1b (Rohr et al., 2001), it is suggested that the Nodal pathway induces early ventral forebrain tissue and the Hedgehog pathway has a role in the maintenance of the hypothalamus. This was suggested by a failure of injected shh sense RNA to rescue nk2.1a expressing tissue in Nodal pathway mutants (Rohr et al., 2001).

6.4.4. Early neuron formation

The next step in patterning of the early fish brain is the appearance of early neurons along the whole length of the neural tube (Ross et al., 1992, Chitnis and Kuwada, 1990, Wilson et al., 1990, Hanneman and Westerfield 1989, Hanneman et al., 1988). The enzyme acetylcholinesterase (Layer, 1983, Wilson et al., 1990) is a good marker for all developing primary neurons, regardless of their function. The neurons appear to develop in the middle of
6. Screening for novel zebrafish mutants

neuromeres in the outer part of the ventral brain wall. Early developing neurons appear to have a close relationship to both the neuromere boundaries and early gene expression patterns. Although the exact temporal sequence of the appearance of these neurons is not crucial for normal brain development, in many cases it does follow a stereotyped pattern (Chitnis and Kuwada, 1990).

Antibody staining can be used to visualise neurons at later stages of development as the pattern of tracts and commissures becomes more complex. From around 14 hours post-fertilisation onwards, the use of either an anti-HNK1 or anti-acetylated tubulin antibody allows the detection of these axon tracts in wholemount embryos. Anti-HNK1 antibody recognises an epitope common to a number of glycoconjugates including cell adhesion molecules (Kruse et al., 1984). It has been suggested that HNK1 is a good marker for early developing axons (Wilson et al., 1990). Anti-acetylated tubulin labels early developing cytoskeletal elements in axons (Piperno and Fuller, 1985, Chitnis and Kuwada, 1990).

6.4.5. Tract formation in the zebrafish

The formation of tracts in the fish brain appears to proceed in a similar way to invertebrate tract formation (Wilson and Easter, 1991). Brain tracts appear to be pioneered by small numbers of neurons extending growth cones. The extended growth cone navigates an environment of columnar epithelial cells to reach its target. Within two days of development thick axon tracts have been formed (Wilson et al., 1990). Careful studies have shown that the initial pioneering growth cones reliably migrate from the same area of the developing brain at a similar developmental time, in different embryos studied. There are several families of proteins that maybe involved in the promotion or inhibition of growth cone extension. These include the Eph family, netrins, semaphorins, cadherins and immunoglobulin superfamily (Kolodkin, 1996, Tessier-Lavigne and Goodman, 1996, Brennan et al., 1997, Wilson et al., 1997). Some of these proteins can act as long-distance guidance cues (for
example netrins). Others are secreted or are found on the cell surface and act in the vicinity of the growth cone (Eph receptor tyrosine kinase signalling molecules, semaphorins, cadherins and immunoglobulins for example).

It has been observed that many axons appear to grow along the boundary between domains of neuroepithelial cells in the CNS. There are two models which may account for this phenomenon. In the first model, both sides of the boundary contain cues which repel the axon and so form a "channel" in the centre down which the extending growth moves. In the second model, one side of the boundary again expresses a repellant cell surface secreted molecule, as well as signalling to the other side of the boundary to control expression of different guidance cues. The character of the induced guidance molecule will determine the direction of growth cone extension. An inhibitory molecule will cause a neat fascicle to grow along the centre of the two boundaries. A cell surface secreted molecule that allows is permissive for growth cone extension will allow the tract to be formed although it will not be neatly fasciculated. Interactions between different guidance molecules can be used to ensure correct axon pathway formation (for review see Wilson et al., 1997).

6.4.6. Formation of commissures in the forebrain

During forebrain development, there are only a few discrete places where axons are able to cross from one side of the neural plate to the other: at 24 hours these include the anterior commissure (AC), posterior commissure (PC), post-optic commissure (POC) and ventral tegmental commissure (VTC). The cells in the midline of the forebrain have a similar cuboidal morphology to cells of the floorplate and may well function in a similar manner.

Pioneering growth cones are first attracted to, and then repelled from, the midline cells in order to form the four commissures. The importance of these cells in the formation of the AC and POC was highlighted by studies of the zebrafish acerebellar (ace) mutants (Shanmugalingam et al., 2000). ace
6. Screening for novel zebrafish mutants

Fish have been shown to have a mutation in the fgf8 gene that leads to incorrect splicing and the generation of a truncated non-functional Fgf8 protein (Reiters et al., 1998). In ace mutants, as well as misspattering of the MHB, there are errors in the patterning of the basal telencephalon and a loss of specification of telencephalic neurons (Shanmugalingam et al., 2000). The AC and POC are seen to be initially absent and then to be fused together at later stages and the optic chiasm is frequently not formed. Cuboidal cells of the anterior midline are mis-specified in ace and so fail to induce expression of midline genes that either influence axon extension (such as net1 and sema3D see Strähle et al., 1997, Halloran et al., 1999) or in genes that can indirectly influence guidance cues such as noi (MacDonald et al., 1997), twhh (Ekker et al., 1995) and six3.

Analysis of the no-isthmus (noi) mutant has also given insights into formation of the commissures in the forebrain (MacDonald et al., 1997). Noi protein is closely related to mouse Pax2 protein. noi zebrafish mutants are seen to show an open choroid fissure in the eye and severe pathfinding defects in axons of the POC and optic chiasm. Noi protein may function to "funnel" axons across the midline in neat fascicles, by activating proteins that inhibit exploration of the growth cone. In noi mutants, the growth cones are seen to enter tissue which no longer expresses Noi protein and although the POC and optic chiasm are formed, they are not neatly fasciculated. Noi has been shown to act downstream of ace/fgf8 (Shanmugalingam et al., 2000) and it is possible that some of the commissural defects seen in ace mutants are attributable to this loss of noi.

A third zebrafish mutant, you-too (yot, Karlstrom et al., 1999) has been found to have defects in commissure formation. yot mutants have no POC, reduced AC and optic nerves that tend to grow dorsally instead of medially. yot has been found to encode a gene with a mutation in gli2, a transcription factor that acts downstream of hedgehog genes (Karlstrom et al., 1999). The midline defects detected in yot embryos may be due to a loss of Hedgehog
signalling through gli2 in the ventral forebrain, which in turn causes a
disruption to the induction of cellular guidance cues in the midline.

6.4.7. Other genes involved in AC and POC formation

Studies of the expression patterns of and in vitro properties of several
other genes has suggested that they may also have roles in controlling
forebrain commissure formation. Expression of shh, L2 and L4 (Wilson et al.,
1997, Brennan et al., 1997) is seen in tissue directly ventral to the POC. Noi
protein (as mentioned above) has been reported in the tissue immediately
dorsal to the POC (MacDonald et al., 1997) and it is likely that the commissure
is formed in the area between these domains of expression. shh has been
shown to regulate Noi protein expression (MacDonald et al., 1997) and so may
control the exact positioning of the POC. The AC also develops between two
domains of gene expression. Tissue ventral to the AC expresses netrin1 and
netrin2, whereas tissue dorsal to the AC expresses rtk1, (Wilson et al., 1997,
Cooke et al., 1997). In a similar way to the POC, the AC appears to develop in
the area of tissue between two domains of gene expression.

6.4.8. Axon scaffold, 24 hours post-fertilisation

At 24 hours post fertilisation the zebrafish brain contains a very simple
set of connected axon tracts, a sort of "axon scaffold", consisting of the
olfactory nerve, four commissures and their associated tracts and three other
tracts. In the accepted nomenclature of zebrafish development, a "tract" is
described as a bundle of axons that course together in the central nervous
system (CNS), without reference to the function, origin or destination of the
axons within it (Wilson et al., 1990). These tracts have been formed by axons
that have grown between the early primary neuron clusters to either form
bundles that connect to one another or to other brain areas.

Early scaffold formation acts to make an orthogonal grid: two
longitudinal tracts are formed (anterior to posterior) with interconnecting
commissures between them (Chitnis and Kuwada, 1990). At 24 hours there
6. Screening for novel zebrafish mutants

is a single prominent axon bundle that lies along the ventral part of the brain, and is continuous along the whole length of the ventral brain. At the anterior end of the brain it passes around the diencephalon to form the postoptic commissure (POC). This longitudinal bundle of axons is called the tract of the postoptic commissure (TPOC). At the level of the midbrain it courses alongside, and slightly dorsal to, the medial longitudinal fasciculus (MLF), an axon pathway which descends the embryo either side of the developing floor plate in the ventral neural tube. Some bundles from the TPOC can be seen to actually join the MLF (Chitnis and Kuwada, 1990). Both tracts continue to course longitudinally through the hindbrain and then on into the spinal cord. The anterior commissure (AC) also passes around the rostral end of the telencephalon, dorsally to the POC. The AC and POC are connected by a small axon tract called the supraoptic tract (SOT). The dorsoventral diencephalic tract (DVDT) joins the TPOC halfway along the length of the diencephalon. The tract of the posterior commissure (TPC) also joins the TPOC, at the boundary between the forebrain and midbrain. The ventral tegmental commissure is found in the floor of the anterior tegmentum and crosses the ventral floorplate in the midbrain. In a similar way to the TPOC, the ventral tegmental commissure is not neatly fasciculated, but instead is seen to be a bundle of fibres crossing the midline (Wilson et al., 1990). A second longitudinal axon tract that arises in the hindbrain and descends the embryo lying parallel to (but not connecting to) the MLF is the lateral line fasciculus (LLF). The LLF is formed from axons of both trigeminal ganglion cells and Rohon-Beard cells (Metcalfe et al., 1990).

6.4.9. Axon scaffold, 32 hours

At 32 hours, the structures that are marked with anti-acetylated tubulin and opsins antibodies (Fig. 6.1.) are very similar to those described for the 24 hour embryo. A lateral view of the embryo shows three commissures (anterior, posterior and postoptic) as well as the supraoptic tract and olfactory placodes (Fig. 6.1A). A different focal plane of the same embryo shows the trigeminal ganglion caudal to the developing eye (Fig 6.1B). Dorsal views of a
6. Screening for novel zebrafish mutants

stained embryo at 32 hours at 10X (6.1C) and 20X magnification (6.1D and 6.1E) show the posterior commissure, epiphysis, lateral lines, medial longitudinal fasciculi and hindbrain commissural interneurons. Rohon-Beard neurons can be seen forming two lines descending the tail. Spinal motor neurons are seen descending orthogonally from the spinal cord neurons towards the yolk sack extension (not shown on diagram). Finally, a ventral view of the anterior embryo shows the anterior commissure, posterior commissure and the interconnecting supraoptic tracts (Fig 6.1F).

The neural network marked by these two antibody combinations is complicated, but does make it possible to screen large numbers of embryos for subtle defects in the network. A summary and description of mutants that were found in the Tübingen 2000 brain screen will be given later in the chapter.

6.4.10. 48 hour scaffold

By two days post fertilisation, there is a dramatic increase in the number of axons in the brain. Most of the newly formed axons appear to join pre-existing tracts formed the day before, rather than making new ones themselves. The tracts are seen to have thickened and to project further than in a 24 hour embryo. After two days of development, the optic nerve has been added, as well as three more commissures and their associated tracts. These include the habenular commissure, the commissure of the posterior tuberculum and a few small dorsal midbrain commissures which form the intertectal commissure (Wilson et al., 1990).

6.5. Screening for novel axon pathfinding mutants

6.5.1. Screen design and protocol

As part of my PhD work, I took part in two different screens for zebrafish mutants. The first screen, at University College London, was designed to allow the identification of genes that are involved in the patterning of the anterior forebrain and in axon guidance. The second screen, at the Max
Fig. 6.1. Anti-acetylated tubulin and anti-opsin antibody staining on 32 hour zebrafish embryos. All panels show anterior to the left except for panel F which is a frontal view with anterior to the top of the image. Panels A and B are lateral views, panels C, D and E are dorsal views. Embryos are about 50mm in length.

Legend: AC, anterior commissure; cb, cerebellum; CNV, trigeminal ganglion; d, diencephalon; HCl, hindbrain commissural interneuron; hb, hindbrain; hy, hypothalamus; LL, lateral line; LLG, lateral line ganglion; mb, midbrain; MHB, midbrain-hindbrain boundary; MLF, medial longitudinal fasciculus; OLF, olfactory placodes; PC, posterior commissure; POC, post-optic commissure; t, telencephalon; TPOC, tract of the post-optic commissure; SOT, supra-optic tract.
6. Screening for novel zebrafish mutants

Planck-Institute for Developmental Biology, Tübingen, identified embryos that had mutations in genes important for correct axon scaffold formation during development. In both screens I screened for novel mutants and helped in re-identification of interesting mutants. The results section of this chapter aims to briefly describe the design and protocols used in these screens. The mutants found in the second screen will be described and specific examples will be shown of both axon pathfinding and morphology mutants.

As part of the UCL screen team, I spent 4 months in Tübingen looking for novel axon guidance mutants. At any one time during the screen the work was shared by two people, who undertook all the tasks associated with finding novel mutants. I helped out in all the tasks ranging from collecting and sorting out embryos, staining embryos with anti-tubulin and anti-opsin antibodies and then screening for mutant phenotypes. Each mutant was then classified on the basis of the severity of its phenotype, the results were entered into a database and the mutants were sent to London or Dresden for re-screening.

6.5.2. UCL screen

The first screen, at University College London, was a small scale screen designed to identify novel zebrafish mutants with either disrupted patterning of the anterior forebrain or axon pathfinding. A cocktail of in situ hybridisation probes that included pax2, emx1 and her1 were used to analyse the patterning of the brain during early development. A morphology screen was performed on live embryos at 3 somites, 24 hours, 48 hours and 72 hours post fertilisation. This was followed by an anti-acetylated tubulin and anti-opsin antibody screen on fixed 48 hour embryos. For a description of in situ hybridisation and antibody staining protocols, see the Materials and Methods section of this thesis.

6.5.3. Tübingen screen 2000

The second screen, for novel brain mutations, was conducted at the Max Planck Insitut für Entwicklungsbiologie, Tübingen, Germany. The screen
6. Screening for novel zebrafish mutants

looked at 32 hour embryos to identify defects in the patterning of the axon scaffold. The mutants were identified using a mixture of antibodies (anti-acetylated tubulin and anti-opsin), to allow visualisation of the axon tracts and the epiphysis. The choice of 32 hours as the optimal stage to identify novel mutations was two-fold: At this age the antibody will penetrate the whole embryos well, without the need for a permeabilisation step in the protocol, making the staining procedure simpler. Secondly, the axon scaffold is still relatively simple, allowing the easy detection of changes in the early "pioneering" brain tracts.

6.6. Results
6.6.1. UCL screen

The UCL screen was very successful. For the screen, 16 mutagenised male fish were crossed to wildtype female fish. Following another incross, a total of 397 F2 families were generated. During the screen, 272 of these families were analysed and a total of 203 novel phenotypes were found that had alteration in a developmental process. In total, about 400 genomes were screened for mutations that affected either nervous system or somite development. Most of these novel mutants exhibited common phenotypes that had been frequently identified in other mutant screens, including cell death, heart oedema, curly down tail, small eyes or paralysis of the embryo. 53 of the novel mutants showed specific phenotypes and of these, 29 had brain phenotypes.

Following on from the screen, and as a collaboration with Corinne Houart's group at Guy's hospital, we are currently working to further characterise several mutant lines indentified in this screen. Mutants that are currently being studied include embryos which exhibit defects in cell differentiation (visage pale), axonal pathfinding (wingnut and shrink), muscle development (akinetto), laterality (heart reversed), cell movements (silberblick) and fin development (no fin).
6.6.2. wingnut

*wingnut* (wig) mutant embryos have a morphological defect in the formation of the optic stalks (Fig. 6.2.). This causes a mis-shaping of the brain to occur and the eyes and brain of the mutant have a characteristic "wingnut" phenotype. Work done by Julie Cooke and Lukas Roth has subsequently shown that these fish have defects in retinal lamination and also in the projection of retinal axons. The retinal axons are seen to frequently project rostrally and ipsilaterally instead of passing caudally and contralaterally (Fig. 6.2.). Innervation of the tectum is also seen to be aberrant. In a collaboration with both Derek Stemple's lab and Will Talbott's group, the mutation is being mapped and the mutation is being sequenced and analysed. It looks possible that *wig* is an unusual allele of the *bashful* gene, although the phenotypes of *bashful* and *wingnut* are significantly different.

6.6.3. akineto

*akineto* is a zebrafish muscle development mutant. It shows a complete loss of movement, although the heart continues to beat normally. Work done predominantly by Pavlina Haramis and Tom Hawkins has shown that although muscle innervation and calcium release in the muscle occurs normally, there is no muscle contraction following stimulation. Myosin fibres are present in the muscles but they fail to form into sarcomeres (Fig. 6.3.). In wild-type zebrafish, electron microscopy reveals that the muscles of the somites are formed into neat sarcomeres which are separated by a point of attachment called the Z membrane. In *akineto* fish, the sarcomeres are not
6. Screening for novel zebrafish mutants

Figure 6.2
Pictures comparing wild-type (A) and (C) and wingnut (wig), (B), (D) and (E) embryos. (A) and (B) are frontal views of live embryos at 18 somites. The arrows mark the size of the eye, which is seen to be reduced in wig embryos. Panels (C), (D) and (E) are dorsal views with anterior to the left. These panels show dil and diO labelled retinal projections in wildtype and wingnut mutant embryos. In wildtype embryos, the projection is entirely crossed whereas in wingnut many axons are ipsilateral (indicated by yellow labelling). The images were kindly provided by Lukas Roth.

Figure 6.3
Akineto is a zebrafish mutant that shows complete loss of movement, although muscle innervation and calcium release following muscle stimulation has been seen to be normal. Panels (A) and (C) are lateral views of wild-type larvae muscle at 48 hours of development. Panels (B) and (D) show akn mutant embryos. (A) and (B) show actin labelling which appears to be relatively normal in akn mutants. Myosin labelling, shows that the large myosin fibres are not properly formed. Panels (C) and (D) show electron microscope images of the muscle organisation, the white arrowheads mark Z-lines in the muscle. In akn mutant embryos, sarcomeres are not properly formed and are not found in parallel to each other. Instead of being seen as neat lines, the sarcomeres are found in random places throughout the muscle. Pictures are kindly provided by Tom Hawkins. Scale bar at bottom of picture relates to the electron microscope images in (C) and (D).
6. Screening for novel zebrafish mutants

organised neatly and show a "brush-like" appearance either side of the Z membrane (Fig. 6.3.). The thicker myosin fibres are seen to be absent. This mutant is currently being mapped in a collaboration with Robert Geisler's lab in Tübingen.

6.6.4. silberblick

One of the mutants isolated in the UCL screen (slb, U148) had a phenotype that is very similar to the silberblick mutant from Tübingen screen 1. slb mutant embryos have conversion-extension defects severe pituitary defects and a mis-positioning of the hypothalamus (Heisenberg et al., 2000b). The slb mutant is known to encode wnt11. U148 did not complement slb mutants and the U148 allele shows a consistently more severe phenotype than those previously isolated. Sequencing of this new slb allele suggests that the mutation affects splicing of the gene, although it is not clear how this generates so severe a phenotype (P. Chandwani, pers. comm.).

Other mutants that have been identified as having brain phenotypes and that are currently being better characterised include gimpy, visage pale, trilobite-like and shrink. A more complete description of the mutants and some pictures of the phenotypes is available at http://www.ucl.ac.uk/zebrafish-group/.

6.7. Tübingen screen 2000

The brain screen identified nearly 500 novel mutants that either showed morphological defects at 24 hours, or defects in tubulin and opsin antibody staining pattern at 32 hours. By the end of the screen 2205 genomes had been screened for novel brain mutant phenotypes. The mutants were rated as being either 1* (fairly interesting, freeze sperm), 2* (interesting, outcross with second priority) or 3* (extremely interesting, outcross with first priority) as they were discovered. The decision of how interesting a mutation was depended on many factors. These included how frequently the phenotype was observed, whether any cell death had been seen in the embryos and how normal the
6. Screening for novel zebrafish mutants
gross morphology was seen to be. Of the 500 identified mutants, 99 were rated either 2* or 3*. They have been outcrossed and are currently being re-identified (Re-Id) to find more carriers of the mutation. The process requires a huge effort and is not yet complete. The results of the Re-Id work have been entered into an online database that allows the mutants to be searched and categorised to make future work with them easier. The database will soon be available on the internet, allowing researchers from other groups to benefit from the brain screen. There are currently 89 mutants that have been entered into the database and will be discussed here. The mutants are included in this thesis as a table with brief descriptions of the phenotype (Fig. 6.4.). Photographs of selected mutant embryos have been chosen and are included in this results section. For more complete descriptions and photographs please refer to the database. The database can be viewed at www.ucl.ac.uk/zebrafish-group/research/GeneticScreens/index.html.

6.7.1. Classes of brain mutant

In order to make further research simpler, the brain mutants isolated in the brain screen have been divided into four different groups on the basis of phenotypic similarities and similarities in axon pathfinding defects. The division into groups is sometimes difficult and may well need to be reassessed after more work has been done to characterise the phenotype and the underlying mutated gene.

The first class of mutants are those that show striking phenotypic similarities to the mutants identified in other zebrafish screens. These include oep, snakehead and cyclops like mutants. The mutants will be tested to see if they complement other known mutants. The second class of mutants are those that show some phenotypic similarities to mutants identified in other zebrafish screens. They include embryos with reduced eye size, curly tails, defects in brain morphology as well as necrosis. The third class contains novel mutants that have not been described before. These range from subtle defects such as a defasciculated tubulin pattern or in the shape of the somites.
<table>
<thead>
<tr>
<th>NAME</th>
<th>TUEBINGEN</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 HC013 - 07</td>
<td>Curled and bent bodies, some only show curled tails. Somites are slightly U-shaped and a retarded jaw. Slow heart beat, reduced pigmentation in eyes and reduced blood circulation.</td>
<td></td>
</tr>
<tr>
<td>1 HC024 - 02</td>
<td>Oop like phenotype at 24 hours.</td>
<td></td>
</tr>
<tr>
<td>1 HJ032 - 02C</td>
<td>Overproliferation of the posterior brain. MHB is disrupted. Looks like a snake head mutant. Cerebellum is also disrupted.</td>
<td></td>
</tr>
<tr>
<td>1 IF056 - 01</td>
<td>No hindbrain or forebrain ventricles, reduced otic vesicle size</td>
<td></td>
</tr>
<tr>
<td>1 IF084 - 02</td>
<td>Strong cyclops phenotype. Curly down tail and protruding jaw. Eyes fused in the middle</td>
<td></td>
</tr>
<tr>
<td>1 IM021 - 02A</td>
<td>Small eyes, flat small head, miss MHB.</td>
<td></td>
</tr>
<tr>
<td>1 IV045 - 04</td>
<td>No eyes, POC not closed, supraoptic tract shows defects.</td>
<td></td>
</tr>
<tr>
<td>1 JI055 - 03A</td>
<td>Embryos lack ant. neural structures. Lack eyes and telencephalon. HB is disorganised, spinal cord is slightly disorganised.</td>
<td></td>
</tr>
<tr>
<td>2 HD014 - 12C</td>
<td>Embryos were headless with a general curly tail phenotype</td>
<td></td>
</tr>
<tr>
<td>2 HE017 - 02A</td>
<td>Dwarf - like, with kinky notochord, blocky somites and bumpy HB.</td>
<td></td>
</tr>
<tr>
<td>2 HE017 - 02C</td>
<td>Dwarf - like, with kinky notochord, blocky somites and bumpy HB.</td>
<td></td>
</tr>
<tr>
<td>2 HE022 - 02A</td>
<td>General brain cell death, no PC, curly down tail. HB and ant. brain fine.</td>
<td></td>
</tr>
<tr>
<td>2 HE409 - 01A</td>
<td>No notochord, head strongly reduced by day 3.</td>
<td></td>
</tr>
<tr>
<td>2 HE409 - 01C</td>
<td>No notochord, head strongly reduced by day 3.</td>
<td></td>
</tr>
<tr>
<td>2 HI090 - 01A</td>
<td>Eyes small and close together. Resembles cyclops in severe cases.</td>
<td></td>
</tr>
<tr>
<td>2 HR168 - 03</td>
<td>Elephant nose. Messy tubulin and HB disorganised.</td>
<td></td>
</tr>
<tr>
<td>2 HS298 - 03A</td>
<td>Cell death in head, ventralised. Flat brain, small eyes, abnormal TPOC.</td>
<td></td>
</tr>
<tr>
<td>2 HS304 - 03A</td>
<td>Reduced staining in the otic vesicle</td>
<td></td>
</tr>
<tr>
<td>2 HU005 - 03A</td>
<td>Smaller forebrain and short body. split tail. Retarded.</td>
<td></td>
</tr>
<tr>
<td>2 HU028 - 02</td>
<td>Deficiencies tissue of the head. Some miss all head tissue, All lack eyes.</td>
<td></td>
</tr>
<tr>
<td>2 HX033 - 09A</td>
<td>No notochord, only one eye in some cases. No Tub. staining ant. of the trigeminal. No evidence of segmentation in the hindbrain.</td>
<td></td>
</tr>
<tr>
<td>2 HX033 - 09B</td>
<td>No notochord, only one eye in some cases. No Tub. staining ant. of the trigeminal. No evidence of segmentation in the hindbrain.</td>
<td></td>
</tr>
<tr>
<td>2 HX033 - 09C</td>
<td>No notochord, only one eye in some cases. No Tub. staining ant. of the trigeminal. No evidence of segmentation in the hindbrain.</td>
<td></td>
</tr>
<tr>
<td>2 HX051 - 03A</td>
<td>Brain necrosis, elephant nose. Tub. shows a lack of AC and messy OB.</td>
<td></td>
</tr>
<tr>
<td>2 HX066 - 01D</td>
<td>Reduced brain vesicles, snakehead - like. No circulation mild oedema.</td>
<td></td>
</tr>
<tr>
<td>2 IB003 - 04</td>
<td>Coloboma, caudal fin expanded in some mutants.</td>
<td></td>
</tr>
<tr>
<td>2 IG430 - 03</td>
<td>Small head, flat hindbrain, MHB difficult to see.</td>
<td></td>
</tr>
<tr>
<td>2 IN171 - 08A</td>
<td>Thin body axis, sSMNs do not project segmentally. Ectopic RBCs in HB. Flattened forebrain.</td>
<td></td>
</tr>
<tr>
<td>2 IN193 - 02A</td>
<td>Large somites in DV axis, short spinal cord. Narrow HB and abnormal neural tissue close to cerebellum.</td>
<td></td>
</tr>
<tr>
<td>2 IO005 - 08C</td>
<td>Severe ant. truncation, no SMNs in the spinal cord; somites look abnormal.</td>
<td></td>
</tr>
<tr>
<td>2 IO005 - 08D</td>
<td>Severe ant. truncation, no SMNs in the spinal cord; somites look abnormal.</td>
<td></td>
</tr>
<tr>
<td>2 IO005 - 08E</td>
<td>Severe ant. truncation, no SMNs in the spinal cord; somites look abnormal.</td>
<td></td>
</tr>
<tr>
<td>2 IO005 - 08F</td>
<td>Severe ant. truncation, no SMNs in the spinal cord; somites look abnormal.</td>
<td></td>
</tr>
<tr>
<td>2 IP113 - 02</td>
<td>Elevated telencephalon, notochord disrupted, no clear MHB.</td>
<td></td>
</tr>
<tr>
<td>3 HF017 - 01A</td>
<td>Disrupted tectum, HB and cerebellum. No MHB. Tubulin shows no SOT. There may be increased Rohon-Beard cells in the spinal cord.</td>
<td></td>
</tr>
<tr>
<td>3 HF017 - 01B</td>
<td>Disrupted tectum, HB and cerebellum. No MHB. Tubulin shows no SOT. There may be increased Rohon-Beard cells in the spinal cord.</td>
<td></td>
</tr>
<tr>
<td>3 HF017 - 01C</td>
<td>Disrupted tectum, HB and cerebellum. No MHB. Tubulin shows no SOT. There may be increased Rohon-Beard cells in the spinal cord.</td>
<td></td>
</tr>
<tr>
<td>3 HF028 - 01A</td>
<td>Bumpy rostral HB, expanded ventricles, small eyes. NT may be wider.</td>
<td></td>
</tr>
<tr>
<td>3 HF140 - 05A</td>
<td>Short trunk, narrow somites, degenerated notochord, bumpy HB, no</td>
<td></td>
</tr>
</tbody>
</table>
MHB. Tubulin messy and incomplete. All tracts present messy.

3 HII68 - 04A MHB abnormal, tectum may be absent. PC faint but present.
3 HJ138 - 03A Enlarged HB vesicle. PC present but messy. Heads look larger than wt.
3 HK013 - 10A AC, MHB and POC are disrupted. Elephant nose. Tectum is disrupted. Small eyes with ventral problems. Kink at the end of tail.
3 HK027 - 03A Ventralised, small body. Spinal neurons are absent or mispatterned.
3 HK027 - 03C Ventralised, small body. Spinal neurons are absent or mispatterned.
3 HK311 - 05 No TPC or epiphysis. Brain appears amorphous. Curly tail down.
3 HK314 - 05A Cell death in head. Some appear to have specific cell death in the HB.
3 HM158 - 05 S-shaped body. Tub. retarded and AC / POC are not closed. Reduced epiphysis and telencephalon.
3 HN148 - 01 Flat head and no MHB folds present. Pax2 staining of MHB is fine.
3 HP023 - 04A No eyes, disrupted tubulin.
3 HS049 - 02 Elephant nose, no AC. HB commisures are disrupted, trigeminal is misspaced. Mild cell death seen.
3 HU519 - 03A Bumpy HB, bent tail. RBCs are scattered, comms. not formed properly.
3 HU519 - 03B Bumpy HB, bent tail. RBCs are scattered, comms. not formed properly.
3 HU519 - 03C Bumpy HB, bent tail. RBCs are scattered, comms. not formed properly.
3 IA015 - 09A Dead head. AC not closed, PC not formed, HB disorganised. Short tail.
3 IS327 - 04A Tub. very disrupted, no scaffold ant. of the SC. No somites obvious.
3 IT419 - 13A No cerebellum. Anterior HB is disorganised, MHB also disorganised.
3 IT419 - 13B No cerebellum. Anterior HB is disorganised, MHB also disorganised.
3 IY001 - 05A Inflated HB, contriction at MHB. Small eyes, shorter body. Elephant nose and mild cell death in the head. Some show unclosed AC.
3 IY001 - 05C Inflated HB, contriction at MHB. Small eyes, shorter body. Elephant nose and mild cell death in the head. Some show unclosed AC.
3 KS015 - 02A Tub. very disrupted. Extra neurons are seen all over the brain. LL appears to originate in the middle of the SC instead of the lateral edge.
3 KS015 - 02B Tub. very disrupted. Extra neurons are seen all over the brain. LL appears to originate in the middle of the SC instead of the lateral edge.
3 HC006 - 04A Retarded. Decreased distance between the AC and PC. HB is malformed.
3 HC006 - 07A Retarded. Decreased distance between the AC and PC. HB is malformed.
3 HE336 - 08 Reduced or no PC. Mild AC defect in the midline.
3 HE416 - 05 No PC, weak opsin staining. Some show weak PC and epiphysis staining.
3 HJ050 - 01 Tubulin staining shows that the AC is not closed.
3 HJ050 - 01A Tubulin staining shows that the AC is not closed.
3 HJ050 - 01B Tubulin staining shows that the AC is not closed.
3 HJ052 - 01A Weak tubulin staining in the supraoptic tract.
3 HK302 - 07 No MHB, rows of cells in the epiphysis are not separated. AC not closed.
3 HL030 - 03 AC and PC thickened with staining between the two. Other tub. fine.
3 HM030 - 04A Reduced or disorganised epiphysis.
3 HM030 - 04B Reduced or disorganised epiphysis.
3 HM079 - 02A Incomplete AC, often seen to be to close to the POC. Pathfinding defects.
3 HM079 - 02B Incomplete AC, often seen to be to close to the POC. Pathfinding defects.
3 HP325 - 03A AC not closed.
<table>
<thead>
<tr>
<th>4</th>
<th>HZ378 - 16</th>
<th>AC not closed. Tubulin is messy, no epiphysis is present.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>IA042 - 02A</td>
<td>Tubulin shows lateral line defect - pathfinding errors in the lateral line.</td>
</tr>
<tr>
<td>4</td>
<td>IF075 - 04</td>
<td>Disorganised epiphysis, PC defasciculated. Disorganised SMNs.</td>
</tr>
<tr>
<td>4</td>
<td>IT704 - 01</td>
<td>CNS is greatly reduced or absent. Seen in a more post, position.</td>
</tr>
<tr>
<td>4</td>
<td>JH055 - 03</td>
<td>AC delayed, TPOC is thicker than normal. HB is also disorganised - lack of comm. neurons.</td>
</tr>
</tbody>
</table>
6. Screening for novel zebrafish mutants

to a complete loss of eyes or an s-shaped body. Group four contains embryos with defects in the tubulin staining pattern that cannot be detected by morphology alone. For example, these include embryos which only show defects in the formation of the commissures and embryos with hindbrain disorganisation. The column on the left hand margin of the results table (Fig. 6.4.) denotes the group which the embryos have been assigned to.

Fig. 6.5. shows two mutants from class 1. The first mutant, HC024-02 shows a curly down tail and reduced head size with small inward rotated eyes that is reminiscent of the Nodal pathway mutant one-eyed pinhead (fig. 6.5B and 6.5B). one-eyed-pinhead (oep) is a mutant that was isolated in the first Tübingen screen and has been shown to encode a member of the EGF-CFC family of proteins and acts as a co-factor with Nodal at the Activin receptor complex (Gritsman et al., 1999). HC024-02 is currently has been tested for complementation and the allele is being sequenced. Test crosses with oep show that it does not compliment and so may encode a new allele of oep. The second example of a group 1 mutant is IF084-02. The embryos look similar to cyclops mutant embryos (Fig. 6.5F), a mutation in another component of the Nodal signalling pathway (Hatta et al., 1991). Tubulin staining of IF084-02 shows that all the commissures appear to be present (Fig. 6.5H). The hindbrain ventricle appears to be reduced in size and the hindbrain tubulin pattern may well be disrupted. Mutant IM021 (Fig. 6.5F) shows a small ventricle above the hindbrain and reduced tissue in the midbrain-hindbrain boundary. The head appears to be slightly reduced in size and the telencephalon protrudes slightly. The mutant looks similar to spiel-ohne-grenzen, a zebrafish midbrain mutant that has been shown to encode a member of the pou2 family of transcription factors (Reim et al., 2002, Burgess et al., 2002, Schier et al., 1996).

Fig. 6.6. shows two mutants selected from class 2, mutants that show some similarities to fish found in other zebrafish mutant screens. HR168-03 (Fig. 6.6B and 6.6D) shows extra tissue in the anterior forebrain, a phenotype
6. Screening for novel zebrafish mutants

Figure 6.5

Live pictures of class 1 mutant embryos. All panels are lateral views with anterior to the left and dorsal to the top. (A), (C) and (E) show wild-type embryos. (B) HC024-02 mutant embryos have a phenotype that is similar to the one-eyed-pinhead mutant. The eyes are reduced and rotated inwards and the whole body axis is smaller than a wild-type sibling. The brain also appears to be reduced. IF084-02 (D) looks similar to a cyclops mutant embryo, with a protruding jaw (arrowhead), small eyes and reduced brain size. Panel (F) shows IM021, which has a small brain, small eyes and lacks a prominent midbrain-hindbrain boundary (arrow, marked MHB).
Figure 6.5.
6. Screening for novel zebrafish mutants

Figure 6.6

Live and antibody stained pictures of class 2 mutant embryos. (A), (C), (E) and (G) are wild-type fish. (B) and (D) show mutant HR168-03 and (F) and (H) show HE409-01 mutants. All panels are lateral views with anterior to the left and dorsal to the top, except for panels (C) and (D) which are dorsal views with anterior to the left. HR168-03 embryos (B) and (D) are stain with anti-acetylated tubulin and anti-opsin antibodies. All the commissures are present in mutants as compared to wild-type (A) and (C), although the instead of forming neat tracts they appear to be disorganised (compare arrowheads and arrows, panels (C) and (D). MHB, midbrain-hindbrain boundary; PC, posterior commissure). HE409-01 mutant have a generally mis-shapen head with an enlarged hindbrain vesicle (hb, arrows in (H) and (G)).
that is sometimes described as "elephant nose". The whole brain morphology appears to be changed, with extra tissue in the hindbrain ventricle. Tubulin staining at 32 hours shows that the commissures are all present but appear to be messy (Fig. 6.6B). Instead of having neat fascicles connecting one side of the brain to the other, the postoptic commissure appears to be joined to the supraoptic tract and to only be loosely bundled. Dorsal views of the hindbrain (Fig. 6.6D) show disorganisation of both the midbrain-hindbrain boundary and the hindbrain commissural neurons. HE409-01 also shows defects in gross brain morphology (Fig. 6.6F and 6.6H). The hindbrain ventricle appears to be enlarged (Fig. 6.6H) and gives the head a more rounded appearance (Fig. 6.6F). As mentioned earlier in this chapter, other zebrafish mutants have been characterised that have defects in the formation of the MHB. acerebellar (ace), spiel-ohne-grenzen (spg) and aussicht (aus) were all isolated in the first Tübingen mutant screen and have disrupted patterning of the MHB. ace has a null mutation in fgf8 (Reifers et al., 1998), spiel-ohne-grenzen has been shown to code for a mutation in POU domain protein pou2 (Reim et al., 2002) and the mutated gene in aus is as yet unknown (Heisenberg et al., 2000). It is possible that HE409-01 has a mutation in a gene related to one of these mutants. A complementation test would be extremely useful in order to assess this possibility.

Fig. 6.7. shows two novel mutants, HX168-07A and IY001-05. Both mutants were identified as part of the 24 hour morphology screen and were assigned to class three. HX168-07A has an increased head size and slightly curly-down tail. The somites are u-shaped instead of being chevron-shaped and the head is enlarged. Many of the curly-down tail mutants isolated in the first Tübingen screen have been shown to have mutations in components of either the Nodal or Hedgehog signalling pathways. As HX168-07A has both a curly-down tail and u-shaped somites it is possible that it may encode a mutation in a gene of the Hedgehog pathway (see Schauerte et al., 1998). IY001-05 has a misshaped head. There is a reduction of tissue in the
Figure 6.7

Figure 6.7 shows two mutants that were isolated as part of the 24 hour morphology screen. Panels (A), (C) and (E) show wild-type embryos, panels (B) and (D) show HX168-07A and panels (F), (G) and (H) show IY001-05 mutants.

All panels have anterior to the left and dorsal to the top, apart from panel (G) which is a frontal view. HX168-07A has a slightly inlarged head, disrupted somite formation (arrows, panels (C) and (D)) and a curly down tail. IY005-01 has a a mis-shapen head and small eyes. There is a reduction of tissue in the midbrain (arrow, panel (H) and the forebrain is extended anteriorly (arrowhead, panel (H)).
6. Screening for novel zebrafish mutants

Figure 6.8

Pictures of class 4 mutants stained with anti-acetylated tubulin and anti-opsin antibodies. (A), (B), (E) and (F) are lateral views with anterior to the left. (C) and (D) show frontal views and (G) and (H) are dorsal views with anterior to the left. HC006-04A mutants (B) and (D) have all commissures in the correct position although the anterior commissure (AC, arrowhead in (C) and (D)) does not close. JH055-03 mutant embryos have a disorganised tubulin pattern. For example, in the area around the midbrain-hindbrain boundary area (arrow marked MHB in panels (G) and (H)) the axon scaffold appears to be very disorganised.
FIGURE 6.8.

A, B: WT and HC006-04A

C, D: WT and HC006-04A

E, F: WT and JH055-03

G, H: WT and JH055-03
midbrain and an extended anterior forebrain. The morphology may well be due to necrosis or cell death in the head.

The fourth class of mutant were those that only showed defects in the anti-tubulin antibody staining pattern. Fig. 6.8. shows two such mutants; HC006-04A and JH055-03. HC006-04A has most of the commissures in the correct place, although the anterior commissure fails to close (Fig. 6.8B and 6.8D). Closing of the commissures is extremely stage variable. The mutants in HC006-04A look to be the same age as wild-type siblings, so it may be a true phenotype. JH055-03 has a disorganised tubulin staining pattern in the midbrain and hindbrain. The axons appear to all be present but not formed into neat fascicles or tracts.

6.8. Discussion

The work undertaken in Tübingen produced a large number of novel fish mutants that showed either morphological defects at 24 hours or defects in the tubulin and opsin antibody staining pattern at 32 hours. The aim of this screen was to identify mutations in single genes that are responsible for correct axon growth cone guidance and axon scaffold formation during zebrafish development. The screen produced 99 mutants that appeared to fulfill this criteria. In some cases the identified mutants were of interest to groups screening for more than one developmental process. For instance, a fish which showed brain defects may also have had an altered heart phenotype or early somite defect and so be of interest to several labs. In these instances the adult fish were re-identified and embryos were divided between each of the interested groups. The 24 hour morphology screen produced a high number of these mutants as we were screening the embryos at an earlier stage than many of the other researchers and so had the first opportunity to spot mutants.

6.8.1. Limitations of the brain screen
6. Screening for novel zebrafish mutants

There are several limitations to screening in general and our brain screen specifically that would limit the number of mutations that were identified as being of potential interest.

The screen for novel brain mutants relied on the ability of the mutated gene to produce an easily detectable phenotype. There may be many developmentally important genes that do not do this and so were not identified during the course of screening. Redundancy of genes during evolution can also act to "mask" the phenotype that would be seen following the loss of function of an important gene. Loss of one gene may facilitate the upregulation of function of another similarly acting gene.

The tubulin and opsin pattern is complicated and marks a large numbers of a developing axon tracts and structures. It is also extremely stage dependant and requires careful staging of embryos to check that a difference is not only due to the age variability in a clutch of embryos. This in practice can act as both a hindrance and a benefit. The complexity on the one hand make screening difficult and slow, but on the other hand allows detection of subtle mutations which may prove to be more interesting in future work. Other groups in the screen 2000 elected for simpler screening protocols which may have been faster, but may have also meant that some important mutations were not identified.

Other limiting factors for large scale genetic screens exist, regardless of the protocol used for the identification of the mutants. The Tübingen screen appeared to have a high rate of mutation (judged by the number of interesting and specific mutants found) and there was plenty of space for the generation of and recovery of novel mutations. Both the amount of aquarium space available and the amount of man power available for setting up fish, collecting embryos, screening for mutations and re-identifying interesting phenotypes did not seem to be limiting. It is my belief that neither of these variables caused serious limitations to the screening process.
6. Screening for novel zebrafish mutants

Problems with the water in the aquarium meant that the fish were not producing as many healthy clutches of embryos as would be ideal and this may have reduced the numbers of mutants found in the screen. The large numbers of adult fish that were produced during the initial ENU treatment regime meant that any fish that did not lay fertilised embryos the first or second time that they were paired had to be discarded. This was necessary to maintain space in the aquarium and nursery, but may have meant that fish carrying interesting mutations were discarded.

Although a total of 2205 genomes were screened, saturation point was not reached for developmentally interesting genes. In a similar way to the first Tübingen screen, it is likely that some genes will have been hit twice. Some genes in the first screen were hit nearly twenty times. Other factors exist that may influence the frequency in which a gene is mutated. Larger genes tend to be hit more often than smaller genes and easy phenotypes are missed less often than other more subtle phenotypes.

6.8.2. Future work

The work undertaken in Tübingen to identify novel brain mutants was only the start of a long process. Much of the work is still being carried out and should eventually help us to further understanding the genes involved in axon scaffold formation during zebrafish development. As a first step, all the mutants have been outcrossed and are currently being re-identified and further characterised using other important markers of brain differentiation during development. The fish are also going to be split into complementation groups. This will determine whether mutants with similar phenotypes encode mutations in the same gene. All the information and mutant descriptions are being entered into a database so that the whole zebrafish community should be able to benefit from the brain screen.
6. Screening for novel zebrafish mutants

The screen for novel brain mutants was very successful and identified over 500 novel mutants, 99 of which had specific brain patterning defects. It is difficult to guess the identity of the genes that have been mutagenised in our novel brain mutants. Further experiments could include testing known brain markers on our mutants. Genes such as \textit{pax2}, \textit{shh} and \textit{noi} and members of the Eph family of signalling molecules (Brennan \textit{et al.}, 1997, Macdonald \textit{et al.}, 1997) have been shown to have a role in the formation of commissures. \textit{In situ} hybridisations with probes for these genes may give further information about the underlying defects that cause a failure of proper axon scaffold formation. All the mutants isolated in the brain screen need to be mapped to give insight into the underlying mutated gene.

The process of axon guidance and scaffold formation is complicated and not yet fully understood. With further work on the characterisation of these mutants, it is my hope that this interesting and highly complex process will be better understood.
Characterisation of monorail, a zebrafish midline mutant

7.1. Abstract
One of the most important processes in the development of the nervous system is the guidance of growing axons from their neuronal cell bodies to correct targets elsewhere in the nervous system or body. One of the signalling centres that has been shown to be important in this process is the floorplate, a band of non-neuronal cells found in the ventral spinal cord. There has been a lot of research showing that the floorplate is an important source of long-range signalling molecules, as well as providing contact-mediated guidance information to growing axons (for review see Kaprielian et al., 2001). Analysis of mutants in a variety of species has shown that the loss or impairment of floorplate function causes developing axons to fail to reach their appropriate targets. In this chapter I characterise a zebrafish floorplate mutant, monorail, that has both floorplate defects and subtle changes in the development of the embryonic axon scaffold.

7.2. Introduction
The signalling pathways involved in the specification and function of floorplate cells during development have been studied extensively and in a wide variety of organisms (see Clarke et al., 1991, Tear et al., 1993, Patten and Plazcek, 2000). Our knowledge is still not complete and there are many questions left to be answered. For example, it is not known how many molecules are involved in axon guidance in the vicinity of the floorplate and the many differences between species are still being addressed and compared. In this chapter I describe the characterisation of monorail (mol), a zebrafish midline mutant with floorplate defects. The phenotype of the mutant is described using a combination of in situ hybridisation probes and antibodies to assess the underlying morphological changes in mol embryos. A candidate gene is
7. Characterisation of monorail, a zebrafish midline mutant

identified using a simple sequence length polymorphism (SSLP) mapping strategy and morpholinos directed against the gene of interest are used to phenocopy the *mol* phenotype. Characterisation of *mol* gives new insight into both the process of specification of the floorplate, induction of ventral CNS neurons and axon guidance at the midline of zebrafish embryos.

7.2.1. Morphology of floorplate cells

The floorplate is a band of non-neuronal epithelial cells found on the ventral floor of the spinal cord, above the notochord. They are morphologically distinct from the other cells of the spinal cord, their cuboidal shape allowing easy recognition using a light microscope (Odenthal *et al.*, 2000). In the zebrafish, the anterior border is found at the level of the ZLI in the diencephalon (Hatta *et al.*, 1991). The width of the band of floorplate cells is different in many species-ranging from an area 15-20 cells wide in rat (Placzek *et al.*, 1993) to a single cell wide in fish. For the purposes of this thesis, the zebrafish floorplate will be used henceforth as the main structure for discussion. The width of the floorplate in zebrafish has been disputed for a long time. Some evidence suggests that it comprises of a single cell band in the ventral midline of the spinal cord (Krauss *et al.*, 1993), whereas other experiments report a three cell wide band comprised of two cell types (Kuwada *et al.*, 1990). Recent work in characterising the floorplate (Odenthal *et al.*, 2000), along with personal observations during my work with *monorail*, favours the latter model with the zebrafish floorplate in the trunk being a three cell-wide structure. Within this three cell wide band there are two distinct cell populations, medial (MFP) and lateral (LFP) floorplate cells. The presence of the two different types of floorplate cells can be assessed by looking at midline marker genes during development. The transcription factor *sonic hedgehog* (*shh*) marks medial floorplate only, whilst *foxa* (also known as *forkhead4*) and *foxa2* (*axial*) mark both medial and lateral cells. Comparative *in situ* hybridisations using these two markers allows the presence of both cell types to be tested in midline mutants.
7. Characterisation of monorail, a zebrafish midline mutant

7.2.2. Induction and development of the floorplate

In the classic model of midline development, ectodermal cells overlying the notochord are induced to from the floorplate by signals that include Shh (for review see Placzek et al., 2000). A population of cells in the node of the chick are seen to express markers found in both the notochord and floorplate. Other node cells are seen to express marker genes only found in the notochord such as Not1/flh and Brachyury/ntl. These cells will eventually end up in the differentiated notochord (Catala et al., 1996). Following incorporation into the midline, the markers are down-regulated and it is not until Shh is secreted from the notochord that floorplate is induced and continues to develop.

However, studies of zebrafish midline mutants have suggested that this classical model may not be correct in all species and that the floorplate may be induced in a slightly different way. Both Hensen's node in the chick and the shield in zebrafish are thought to contain a group of midline precursor cells (MPCs) that are already committed to form either notochord or floorplate (for review see Le Douarin and Halpern, 2000). Genes such as the transcription factor no tail, shh or HNF3β may be involved in the commitment to form either notochord or floorplate. The zebrafish has two hedgehog genes, both of which are expressed in the same cells in the shield (Ekker et al., 1995). During convergence-extension, cells fated to form notochord retain shh expression, whilst floorplate cells predominately express twhh. In summary, rather than being induced by signals from the underlying notochord, this second model of floorplate specification suggests that division of MPCs occurs early on during organiser patterning (Le Douarin and Halpern, 2000). Further evidence for this hypothesis comes from analysis of the zebrafish no tail and floating head mutants. Following a mis-specification of dorsal mesendodermal cells the notochord fails to form, but the overlying floorplate is correctly formed. (Halpern et al., 1997, Talbot et al., 1997). The role of the differentiated notochord in floorplate induction in fish may not be as critical as was first suggested in the classic model.
In contrast to work undertaken in other species, analysis of midline mutants suggest that the formation of MFP and LFP in zebrafish is sequential and involves two different signalling pathways. The Nodal signalling pathway provides cues for the development of MFP cells. cyclops (cyc) and oep mutants both have a complete lack of floorplate (Sampath et al., 1998, Strähle et al., 1997), with a reduction of all floorplate markers in mutant embryos. Injection of cyc RNA into cyc mutants has been shown to rescue the fused eye phenotype and increase the number of shh expressing cells in the ventral brain and neural tube (Sampath et al., 1998) providing further evidence for the Nodal pathway acting upstream of the Hedgehog pathway in midline development. Later on in development, the MFP has been seen to recover in both cyc and oep mutants (Strähle et al., 1997). This recovery of phenotype has been shown to be due to temporally regulated expression of shh that induces MFP formation in the absence of cyc gene function (Carl Neuman, pers. comm.).

On the other hand, Hedgehog pathway mutants including sonic-you (syu) lack LFP, although the MFP develops normally (Schauerte et al., 1998). Expression of LFP markers including foxa and foxa2 are reduced in syu embryos, with expression being limited to the MFP cells. This combination of mutant analysis has led to a suggestion that the zebrafish floorplate is formed sequentially. Nodal signalling early during midline patterning induces the formation of the MFP. LFP cells are then induced by signalling activity emanating from either the notochord or the MFP cells (Odenthal et al 2000). Mutants in the Hedgehog pathway display normal MFP development, while LFP induction does not occur (Schauerte et al., 1998). Following formation, floorplate cells express differentiation markers including HNF3β/foxa2 and col2a1, allowing them to function in patterning the ventral neural tube.

7.2.3. Characterisation of zebrafish mutants
7. Characterisation of monorail, a zebrafish midline mutant

A lot of the genes involved in floorplate development in zebrafish were identified by the characterisation of mutant embryos that lack a properly developed floor plate. The next section will briefly describe these various zebrafish floorplate mutants. One of the strengths of the zebrafish as a developmental model is the number of mutations that have been found in genes that are important for normal development. By characterising the phenotypes of these mutants and linking the phenotype to the mutated gene, it is possible to dissect out pathways important for normal development. Mutations can be induced using ENU, a chemical mutagen that causes point mutations in the sperm of male founder fish. The fish are outcrossed through two generations and then inbred to screen for changes in the tissue or organ system of interest.

7.2.4. The midline group of zebrafish mutants

Tissues of the midline such as the floorplate and the notochord have long been known to be important for the correct patterning of the ventral neural tube and somites. The first Tübingen screen in 1996 found a group of mutant fish that showed defects in the formation of the floorplate, and these fish have been instrumental in the further elucidation of normal midline development (see Brand et al., 1996). The mutants in this group all show abnormal body shape, with a distinct curly down tail. Other phenotypes that are seen include u-shaped somites, cyclopia, abnormal optic nerve projections and a reduction in total body size. Of 113 midline mutations that were found in the screen, 13 were selected for detailed analysis as they showed a defect in floorplate development (Brand et al., 1996). These mutants include cyclops, squint, one-eyed pinhead, schmalspur, chameleon, detour, iguana, you-too, sonic-you, you, uboot, schmalhans and monorail. In this introduction, each mutant will be described in parallel with the signalling pathway which it is known to be defective in.

7.3. The Nodal signalling pathway
7.3.1. Introduction
7. Characterisation of monorail, a zebrafish midline mutant

Nodal is a TGFβ related signalling factor that is known to have a role in the induction of the mesoderm, patterning of the anterior-posterior axis and the specification of left-right asymmetry (see Schier and Shen, 2000). Nodal signalling is mediated by a dimerised pair of Activin-like receptors which in turn activate members of the Smad family of transcription factors. The complex of Smad2 and Smad4 binds to Schmalspur (also known as FAST/FoxH1) in the nucleus of the target cell to activate gene transcription (Fig. 7.1). The Nodal family has two related genes in zebrafish, *squint* and *cyclops*. Double mutants for both of genes (ie *sqt:cyc*) show a lack of head and trunk mesendoderm and a misplaced anterior-posterior axis; evidence for a loss of Nodal signalling activity. For a review of Nodal signalling please refer to Schier and Shen, 2000.

7.3.2. Other factors in the Nodal pathway

In addition to Activin receptors, Nodal signalling is modulated by a variety of extracellular or membrane bound factors.

Cerberus is an antagonist of Nodal, Wnt and BMP (Piccolo *et al*., 1999, Bouwmeester *et al*., 1996). It is an extracellular protein that is a member of the cysteine-knot superfamily. Cerberus acts as a regional specific inhibitor that blocks BMP and Wnt as well as Nodal, although a truncated form of Cerberus ("Cer-short") exists that blocks Nodal activity only. Lefty belongs to a subfamily of the TGFβ superfamily of secreted signalling proteins. Lefty protein lacks an important long α helix motif and cysteine residue that are known to be critical in stabilising other homodimer and heterodimer TGFβ complexes (Meno *et al*., 1996). Lefty antagonises Nodal by competing for the same binding site on the Activin receptor complex, overexpression showing exactly the same phenotype as a *sqt:cyc* double mutant. This competitive block of Nodal can be counteracted by overexpressing a mixture of *cyclops* and *squint* (Bisgrove *et al*., 1999, Meno *et al*., 1996). Finally, *oep* is a member of the EGF-CFC gene family that also includes the mouse genes *cripto* and *cryptic*. All members of the family encode membrane associated proteins containing an epidermal growth
Fig 7.1., simplified diagram of the Nodal signalling pathway. Nodal is inhibited by both Lefty and Cerberus. Oep (also called EGF-CFC) acts as a co-factor of Nodal. Activation of a type I and II activin receptor dimer causes phosphorylation of smad2 and the formation of a smad2-smad4 complex. This in turn activates transcription factors in the nucleus of the target cell, including the FAST family of transcription factors. For a complete description of the Nodal pathway, see the text in the introduction.
thought to be caused by a failure of to maintain the structure and function of the developing midline. The zebrafish sqint (sqt) mutant is known to encode another Nodal related protein. sqt mutant embryos have a variable phenotype. Some are cyclopic and completely lack notochord. Others have a wild-type notochord and well separated eyes (Feldman et al., 1998). In a similar way to cyc, sqt mutants have defects in the prechordal plate and ventral neural tube. Recent research shows that sqt can function as a morphogen, while cyc cannot (Chen and Schier, 2001). sqt can function at a distance to initiate transcription of downstream genes in a concentration and distance related response, with no intermediate relay mechanism necessary to mediate its actions (Chen and Schier, 2001). sqt:cyc double mutants have a much more severe phenotype than either cyc or sqt alone and look similar to oep mutants (see next section). Both sqt and cyc mutants show an absence of floorplate tissue, with the loss of foxa2 expression.

7.3.5. one-eyed pinhead

one-eyed pinhead (oep) is another gene acting in the Nodal signalling pathway and is known to be co-factor at the activin receptor complex that is activated by Nodal. Oep is a member of the EGF-CFC family of proteins and is required for the formation of ventral neurectoderm, prechordal plate and the organiser (Gritsman et al., 1999). Oep signalling in the zebrafish has both a maternal and a zygotic component. Embryos lacking both maternal and zygotic Oep have been seen to have a more severe phenotype than embryos lacking only one source. oep mutants have no floorplate, there is a reduction of anterior neural tissue and the eye anlage is fused together at the front to form a single eye field (Brand et al., 1996, Schier et al., 1997). Mutants lacking both sources of oep (called maternal -zygotic oep) show a more severe phenotype with defects in the positioning of the anterior-posterior axis (Gritsman et al., 1998, Strahle et al., 1997).
7. Characterisation of monorail, a zebrafish midline mutant

7.3.6. schmalspur

_schmalspur_ (sur) is the final mutant in the group that completely lacks floorplate tissue. The midbrain and hindbrain both have reduced tissue and the eyes are slightly closer together than in wild type siblings (Brand _et al._, 1996). Defects in the prechordal plate and ventral neurectoderm are also seen (Pogoda _et al._, 2000). In a similar way to oep, the sur gene has both a maternal and a zygotic component. In mutants lacking both sources of sur, the phenotype is much more severe, a morphologically distinct organiser is never formed and all axial structures are malformed. _sur_ embryos also show defects in dorsal midline cell types in the establishment of left-right asymmetry (Sirotkin _et al._, 2000). The mutated gene in _sur_ embryos has been cloned and is known to encode a winged-helix forkhead family transcription factor called FoxH1 or FAST1 (Pogoda _et al._, 2000, Sirotkin _et al._, 2000). FAST1 acts through a smad2 / smad4 complex in the Nodal signalling pathway. FAST1 is necessary for correct Nodal pathway function and also acts in a feedback loop to modulate Nodal activity.

In summary, the observation that oep embryos lack all floorplate cells at early developmental stages suggests that Nodal signalling is necessary for initial floorplate development (Strahle _et al._, 1997). Axial mesendoderm expression of _shh_ in oep mutants is not changed as compared to wild-type embryos, suggesting that signalling through the Hedgehog pathway may act synergistically with the Nodal pathway to specify the midline neuroectoderm. In support of this model, the Nodal signalling pathway acts cell autonomously to induce the expression of Hedgehog pathway proteins (Muller _et al._, 2000). The role of the Hh pathway in LFP cell formation will be discussed in the next section.

7.4. The Hedgehog signalling pathway

7.4.1. Introduction
7. Characterisation of monorail, a zebrafish midline mutant

The Hedgehog family of signalling molecules was first identified in *Drosophila*. *Hh* is a segment polarity gene that is important for the correct patterning of developing body segments. *Drosophila* embryos with a mutation in the gene die at the end of embryogenesis, and secrete a larval cuticle that is covered in spiky denticles. The resulting embryo looked like a hedgehog and the gene was thus named (Perrimon and Mahowald, 1987, Tabata et al., 1992). Hedgehog is now known to be a morphogen that has both short and long range effects. During *Drosophila* embryogenesis, Hedgehog affects a wide range of processes including segmentation, wing formation, leg and eye patterning as well as correct brain formation (McMahon, 2000).

In contrast to *Drosophila*, vertebrates have multiple copies of the *hedgehog* gene. Zebrafish, for example, have three genes called *Sonic*, *Echidna* and *Tiggywinkle hedgehog* respectively (Krauss et al., 1993, Currie et al., 1996, Coutelle et al., 2001). The vertebrate Hedgehog family has been implicated in a wide variety of developmental processes, including the patterning of the ventral neural tube, cartilage differentiation and limb bud patterning. Hedgehog signalling has been shown to constitute an early wave of patterning in the developing retina, specifying the proximo-distal axis of developing eyes (Neumann and Nüsslein-Volhard, 2000). Changes in Hedgehog signalling have also been linked to tumour formation throughout the body (Ruiz I Altaba et al., 2002). These tumour types include basal cell carcinomas and neuroectodermal tumours.

7.4.2. Short range signalling in the Hedgehog pathway

One of the more surprising findings about short range signalling in the Hedgehog pathway was that cholesterol is needed to facilitate trafficking of the Hedgehog (*Hh*) molecule out of the cell (Ingham and McMahon, 2001 and references therein). *Hh* signalling is usually explained in terms of activity between two adjacent cells. In the first cell the *Hh* molecule is cleaved to form two smaller C terminal and N terminal fragments. The function of the C terminal
7. Characterisation of monorail, a zebrafish midline mutant

fragment is not yet known. Conversely, the N terminal fragment binds cholesterol and is sufficient for all known Hh signalling activity. The resulting complex undergoes another palmitoylation and is then trafficked to the surface of the cell where it acts on the adjacent cell. The cleavage of Hh is an autocatalysed process and in the absence of cholesterol, the activity of Hh is inhibited (McMahon, 2000). The sequestered Hh and cholesterol complex acts on the cell membrane of the second cell by binding and inhibiting a protein called Patched. In the "normal" state of events, Patched is found bound to and inhibiting another protein, Smoothened (a seven pass G-protein coupled receptor, see Murone et al., 1999). The inhibition provided by Patched is removed by the inhibitory action of the Hh fragment. Smoothened in turn acts indirectly to regulate the activity of members of the gli family of genes (from glioblastoma) in the nucleus of the target cell (Murone et al., 1999). Gli proteins are zinc finger transcription factors that can bind DNA at specific amino acid sequences and change downstream effects (Fig. 7.2). The zebrafish has three gli genes that can act downstream of Hedgehog signalling. gli1 has been show to act as an activator gene and is able to induce the expression of various midline marker genes including axial (foxa2) and shh (Hynes et al., 1997). gli3 is a repressor of Hh signalling and inhibits all downstream effects of gli1 (Ruiz i Altaba, 1998). On the other hand, gli2 has an intermediate role. In some situations it acts as an activator gene and in other cases it acts to repress Hh signalling (Matise et al., 1999). These changes in the activation of various transcription factors can be viewed as a mechanism for the downstream function of the Hh pathway.

7.4.3. Long range signalling in the Hedgehog pathway

There is now a large amount of evidence that Hedgehog can act as a morphogen in both the ventral neural tube and the Drosophila wing imaginal disc (Poole et al., 1985, Ingham and McMahon, 2001). In the ventral neural tube, Hedgehog protein is secreted from both the floorplate and notochord to specify cell identity. The protein forms an "activity gradient" with the highest
Figure 7.2., simplified diagram of the Hedgehog signalling pathway. The Hedgehog is released from the sending cell and acts to decrease the repression of Smoothend by Patched. Patched activates Gli genes via several downstream targets (including fused etc). Two forms of Gli exist; Gli repressor and Gli activator. Gli in turn continues to either activate or inhibit downstream gene transcription in the nucleus of the target cell. These targets include both Gli and Patched. This is a simplified diagram and is mostly based on work done in Drosophila. Diagram is adapted from McMahon and Ingham, 2001.
7. Characterisation of monorail, a zebrafish midline mutant

The Hedgehog family of signalling molecules was first identified in *Drosophila*. *Hh* is a segment polarity gene that is important for the correct patterning of developing body segments. *Drosophila* embryos with a mutation in the gene die at the end of embryogenesis, and secrete a larval cuticle that is covered in spiky denticles. The resulting embryo looked like a hedgehog and the gene was thus named (Perrimon and Mahowald, 1987, Tabata *et al.*, 1992). Hedgehog is now known to be a morphogen that has both short and long range effects. During *Drosophila* embryogenesis, Hedgehog affects a wide range of processes including segmentation, wing formation, leg and eye patterning as well as correct brain formation (McMahon, 2000).

In contrast to *Drosophila*, vertebrates have multiple copies of the *hedgehog* gene. Zebrafish, for example, have three genes called *Sonic*, *Echidna* and *Tiggywinkle hedgehog* respectively (Krauss *et al.*, 1993, Currie *et al.*, 1996, Coutelle *et al.*, 2001). The vertebrate Hedgehog family has been implicated in a wide variety of developmental processes, including the patterning of the ventral neural tube, cartilage differentiation and limb bud patterning. Hedgehog signalling has been shown to constitute an early wave of patterning in the developing retina, specifying the proximo-distal axis of developing eyes (Neumann and Nüsslein-Volhard, 2000). Changes in Hedgehog signalling have also been linked to tumour formation throughout the body (Ruiz I Altaba *et al.*, 2002). These tumour types include basal cell carcinomas and neuroectodermal tumours.

7.4.2. Short range signalling in the Hedgehog pathway

One of the more surprising findings about short range signalling in the Hedgehog pathway was that cholesterol is needed to facilitate trafficking of the Hedgehog (Hh) molecule out of the cell (Ingham and McMahon, 2001 and references therein). Hh signalling is usually explained in terms of activity between two adjacent cells. In the first cell the Hh molecule is cleaved to form two smaller C terminal and N terminal fragments. The function of the C terminal
7. Characterisation of monorail, a zebrafish midline mutant

fragment is not yet known. Conversely, the N terminal fragment binds cholesterol and is sufficient for all known Hh signalling activity. The resulting complex undergoes another palmitoylation and is then trafficked to the surface of the cell where it acts on the adjacent cell. The cleavage of Hh is an autocatalysed process and in the absence of cholesterol, the activity of Hh is inhibited (McMahon, 2000). The sequestered Hh and cholesterol complex acts on the cell membrane of the second cell by binding and inhibiting a protein called Patched. In the "normal" state of events, Patched is found bound to and inhibiting another protein, Smoothened (a seven pass G-protein coupled receptor, see Murone et al., 1999). The inhibition provided by Patched is removed by the inhibitory action of the Hh fragment. Smoothened in turn acts indirectly to regulate the activity of members of the gli family of genes (from glioblastoma) in the nucleus of the target cell (Murone et al., 1999). Gli proteins are zinc finger transcription factors that can bind DNA at specific amino acid sequences and change downstream effects (Fig. 7.2). The zebrafish has three gli genes that can act downstream of Hedgehog signalling. gli1 has been shown to act as an activator gene and is able to induce the expression of various midline marker genes including axial (foxa2) and shh (Hynes et al., 1997). gli3 is a repressor of Hh signalling and inhibits all downstream effects of gli1 (Ruiz i Altaba, 1998). On the other hand, gli2 has an intermediate role. In some situations it acts as an activator gene and in other cases it acts to repress Hh signalling (Matise et al., 1999). These changes in the activation of various transcription factors can be viewed as a mechanism for the downstream function of the Hh pathway.

7.4.3. Long range signalling in the Hedgehog pathway

There is now a large amount of evidence that Hedgehog can act as a morphogen in both the ventral neural tube and the Drosophila wing imaginal disc (Poole et al., 1985, Ingham and McMahon, 2001). In the ventral neural tube, Hedgehog protein is secreted from both the floorplate and notochord to specify cell identity. The protein forms an "activity gradient" with the highest
Figure 7.2., simplified diagram of the Hedgehog signalling pathway. The Hedgehog is released from the sending cell and acts to decrease the repression of Smoothend by Patched. Patched activates Gli genes via several downstream targets (including fused etc). Two forms of Gli exist; Gli repressor and Gli activator. Gli in turn continues to either activate or inhibit downstream gene transcription in the nucleus of the target cell. These targets include both Gli and Patched. This is a simplified diagram and is mostly based on work done in Drosophila. Diagram is adapted from McMahon and Ingham, 2001.
concentration ventrally. The protein acts cell non-autonomously and can specify the formation of different cell types depending on the concentration of Hedgehog perceived. Cells requiring higher concentrations of Hedgehog for specification are found nearer to the midline than those which require lower concentrations (Placzek et al., 1995, McMahon and Ingham, 2001). Acting in concert with the Retinoid, Nodal and BMP signalling pathways (Pierani et al., 1999, Muller et al., 2000, Barth et al., 1999), Shh acts to induce 5 different subtypes of ventral neurons: V0, V1, V2, V3 and somatic motorneurons (sMNs).

The neurons found in the ventral spinal cord predominantly participate in motor output, whereas those in the dorsal spinal cord process and relay sensory information. The induction of the correct neuronal type at different dorso-ventral levels is dependant on the secretion of Shh from the axial midline (Briscoe and Ericson, 2001). Incremental two to three fold increases of secreted Shh concentration results in the induction of the 5 different types of ventral neurons. The current model describing how the differential expression levels of Shh protein are interpreted suggests that a graded Shh signal controls the expression of homeodomain proteins to establish five distinct domains of progenitor cells (for review see Briscoe and Ericson, 1999 or Briscoe and Ericson, 2001). Cross-repressive interactions between the homeodomain proteins are thought to maintain and refine the boundaries between the progenitor domains.

The homeodomain proteins induced by Shh can be divided into two main classes. Class I proteins, including Pax6 and Dbx2, are repressed by Shh and so act to define the ventral limit of the progenitor domain. Conversely, Class II proteins such as Nkx2.2 and Nkx6.1 are dependant on Shh for their expression and therefore the dorsal limit of their expression defines the dorsal limit of the progenitor domain (Briscoe et al., 2000). It is not yet clear whether Shh acts to directly repress or activate these proteins, or whether Shh acts by signalling through only one class of homeodomain protein, which can in turn affect the
7. Characterisation of monorail, a zebrafish midline mutant

expression of the other protein class. Studies in chick have shown that ectopic overexpression of individual homeoproteins can cause a switch in neuronal fate (see Briscoe and Ericson, 2001). An example of this is the specification of the three most ventral neuron types in the spinal cord. The V2, sMNs and V3 are induced by a combination of Nkx6.1, Nkx2.2 and Irx3 proteins. Nkx6.1 acting in the absence of Irx3 induces sMNs generation. The combined expression of Nkx6.1 and Irx3 induces V2 neurons, whereas the combined expression of Nkx6.1 and Nkx2.2 induces V3 neurons. The homeodomain proteins also activate the expression of neuronal subtype determining genes and differentiation genes, including \textit{Lim3} and \textit{Isl1} (Pfaff \textit{et al.}, 1996, Sharma \textit{et al.}, 1998). It is not known how the specification of these neuronal subtypes at different dorso-ventral levels is integrated with the regulation of neurogenesis. One possibility is that the homeodomain proteins activated by Shh can in turn restrict the expression of neurogenic genes such as \textit{notch} and the \textit{delta} family of genes (Briscoe and Ericson, 2001).

7.4.4. Zebrafish Hedgehog pathway mutants provide insights into the activity of Hedgehog in patterning the central nervous system

Several zebrafish mutants have been isolated that have been found to have defects in midline patterning. Many of these mutants are now well characterised and the mutated genes have been cloned and identified.

7.4.5. \textit{smoothened}

The zebrafish \textit{smoothened} mutant is particularly interesting as all Hedgehog signalling acts through \textit{smoothened} and this therefore provides a "bottle-neck" in the pathway. \textit{smoothened} mutants lack all, or nearly all, Hh signalling and the phenotype of \textit{smoothened} (\textit{smu}) mutant embryos is consistent with a severe disruption of the Hh signalling pathway during development. The embryos show U-shaped somites with no horizontal myoseptum, a reduced floorplate, mild cyclopia and a posteriorly curved body (Chen \textit{et al.}, 2001, Varga \textit{et al.}, 2001). The head has severe craniofacial
defects and the pectoral fin buds lack endoskeletal discs and actinotrichs (see syu, Neumann et al., 1999). As would be predicted for a mutation in the Hh pathway, smo mutants lack LFP cells but show normal MFP development.

7.4.6. sonic-you

The zebrafish mutant sonic-you (syu) is known to be caused by a mutation in the shh gene (Schauerte et al., 1998). The homozygous embryos lack lateral floorplate cells and show defects in somite patterning, pectoral fin buds and motor neuron axon pathfinding. The motility of the embryos is reduced, due to the disruption of muscle fibre patterning. The formation of medial floorplate cells in these mutants was one of the key pieces of data used to show that specification of the two populations of fish floorplate cells were linked to the activities of two separate signalling pathways (Schauerte et al., 1998, Odenthal et al., 2000). Cranial branchiomotor neurons are seen to be reduced in the hindbrain of syu mutants (Bingham et al., 2001, see below). syu mutant embryos have been extremely useful in the elucidation of the function of the shh gene during development. The comparatively weaker phenotype of syu fish as compared to shh mutants in other species demonstrates the redundancy that is characteristic of the fish Hh pathway. For example, mouse mutants for the shh gene show a complete loss of floorplate, lack motorneurons in the neural tube and have a single fused optic vesicle instead of bilateral eyes (Chiang et al., 1996).

Other mutants with midline defects have been found to encode members of the gli family of genes. As discussed in the introduction to this section, gli genes are transcription factors that act as transducers of the Hh signal. Analysis of mutants in which the function of one of the gli genes has been reduced has made the study of the different roles of gli genes during development simpler.

7.4.7. detour
Embryos with mutations in the zebrafish detour (dtr) gene show a partial floorplate phenotype, with lateral floorplate cells missing but medial floorplate present. They have a reduced ventral CNS, no abducens motor nucleus (cranial nerve VI) and eyes that are turned in and have abnormal retinotectal projections (Karlstrom et al., 1996). The number of motor neurons in the hindbrain and midbrain of dtr are greatly reduced, whilst spinal motor neurons are unaffected (Chandrasekhar et al., 1999). There is a large reduction, or even absence, of all the nuclei that usually innervate the gills and jaw muscles. Analysis of early markers for patterning (including krox20, valentine and rtk1/EphA4 show that early brain development is normal. Expression of hh genes in the floorplate is also normal in mutant as compared to WT (Chandrasekhar et al., 1999). The dtr gene acts cell autonomously downstream of protein kinase A in the Hedgehog pathway, in order to specify the induction of branchiomotor neurons in the hindbrain and midbrain (Chandrasekhar et al., 1999). The normal patterning seen in the spinal cord of dfr mutants suggest that there may be a dtr -like gene that works in a similar way in the spinal cord. Analysis of the dtr mutant provides evidence that the Hh pathway may signal through different downstream genes at different anterior-posterior positions along the spinal cord. The identity of the dtr gene is not yet published although it is reported to be gli1 (Rolf Karlstrom, personal communication).

7.4.8. you-too

you-too (yot) is another member of the group of mutants that only have a medial floorplate. The eyes are turned in towards the midline and the mutants have abnormal retinotectal projections (Brand et al., 1996). The somites of yot are u-shaped, lack a horizontal myoseptum and slow muscle fibres are not formed (Du and Dienhart 2001, van Eeden et al., 1996). Close observation of the growing axons of the eye show ipsilateral projections instead of the normal contralateral axon tract (Karlstrom et al., 1996). The ventral forebrain is abnormally patterned. The gene encoding the yot phenotype has now been cloned by synteny and is known to be gli2 (Karlstrom et al., 1999. yot mutants
7. Characterisation of monorail, a zebrafish midline mutant

contain a copy of the gli2 gene that has a mutation resulting in C terminal truncated proteins (Karlstrom et al., 1999). Characterisation of yot gives an example of the three zebrafish hh homologues acting in a similar pathway to pattern myogenesis. You-too embryos fail to form adaxial myocytes when either shh, twhh or ehh are ectopically overexpressed showing that Gli2 is needed downstream of all these genes (Du and Dienhart, 2001). At the moment there is no known mutant for gli3.

Other mutants with midline phenotypes have been found, although the underlying mutated genes have not yet been identified. One of these mutants, chameleon (con), has a phenotype that is similar to that of dtr. Con mutants have only a medial floorplate, a reduced ventral CNS with the trochlear motor nucleus being completely absent, and eyes that are turned in towards the midline (Karlstrom et al., 1996). Con has a curled body phenotype, suggestive of con being a Hh Hedgehog or Nodal pathway mutant. Analysis of the growing optic nerve shows that con embryos have ipsilaterally growing optic nerves (instead of contralateral in wild-type) and axonal pathfinding mistakes within the eye (Karlstrom et al., 1996).

7.4.9. Expression pattern of the Hedgehog ligands in midline tissue during development

7.4.9.1. sonic hedgehog

The earliest expression of sonic hedgehog (shh) in the zebrafish is seen in the shield at 60% epiboly. Expression during convergence-extension and early somitogenesis is seen in the mesendoderm that will form the notochord and floorplate. Later on in development, expression is seen in the notochord, floorplate and in the ventral part of the rostral forebrain, with an extension of expression dorsally in the diencephalon (Concordet et al., 1996, Fietz et al., 1994). Subsequently shh expression is reduced in the notochord but maintained in the floorplate (Fietz et al., 1994). Expression in the brain and floorplate are maintained throughout development.
7. Characterisation of monorail, a zebrafish midline mutant

7.4.9.2. tiggywinkle hedgehog

The expression of \textit{tiggywinkle} (\textit{twhh}) during zebrafish development is similar, but not identical, to that of \textit{shh} (Ekker \textit{et al.}, 1995). The expression of \textit{twhh} precedes that of \textit{shh}, \textit{twhh} first being detected at around 50% epiboly in a portion of the shield. After convergence-extension \textit{twhh} expression is seen in the midline mesoderm, the precursor of the notochord. The expression in the brain is similar to that of \textit{shh}: both genes are expressed in a discrete area of the ventral diencephalon between the two future optic stalks. Expression of \textit{twhh} in the midline becomes restricted to the neural tube from tailbud stages onwards, whereas \textit{shh} is expressed in the notochord as well.

7.4.9.3. echidna hedgehog

The third member of the zebrafish Hedgehog family to be found was \textit{echidna hedgehog} (\textit{ehh}). Expression of \textit{ehh} during fish development is only seen in the notochord with, particularly strong expression in the tail. The eyes and brain show no expression at all.

7.4.9.4. Table of hedgehog gene expression during development

<table>
<thead>
<tr>
<th></th>
<th>60% epiboly</th>
<th>tailbud</th>
<th>24hr</th>
<th>48hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{shh}</td>
<td>mesendoderm and ventral brain</td>
<td>notochord, floorplate and ventral brain</td>
<td>notochord, floorplate and ventral brain</td>
<td>notochord, floorplate and ventral brain</td>
</tr>
<tr>
<td>\textit{twhh}</td>
<td>mesendoderm and ventral brain</td>
<td>notochord, floorplate and ventral brain</td>
<td>floorplate and ventral brain only. Not notochord</td>
<td>floorplate and ventral brain but not notochord</td>
</tr>
<tr>
<td>\textit{ehh}</td>
<td>no expression</td>
<td>notochord and MHB</td>
<td>caudal notochord but not tailbud</td>
<td>caudal notochord but not tailbud</td>
</tr>
</tbody>
</table>
7. Characterisation of monorail, a zebrafish midline mutant

7.4.10. Redundant roles of Hedgehog signalling in midline patterning

One of the problems facing researchers analysing the Hh family in zebrafish is the large amount of redundancy seen in the Hh pathway. The three known zebrafish family members have overlapping domains of expression and complementary functions. Removal of one of the ligands can simply allow the upregulation of another, or the recovery of function by another family member (Ingham and McMahon, 2001). Studies on the zebrafish sonic-you mutant (which lacks shh signalling, Schauerte et al., 1998), combined with morpholinos designed against twhh and ehh, have allowed the patterning of the midline to be studied in the absence of total Hh pathway signalling. The medial floorplate is still formed in embryos which lack nearly all Hh signalling (Lewis and Eisen, 2001, Etheridge et al., 2001). However, induction of motorneurons is more dependent on Hh signalling. Loss of any one zebrafish Hh protein does not affect formation of motorneurons, suggesting that any two Hh proteins are sufficient for this induction. ehh is less efficient than shh and twhh in inducing motorneurons, which may be related to differing temporal and spatial expression between the three genes (Lewis and Eisen, 2001). shh and twhh have also been shown to induce all branchiomotor neurons in the developing zebrafish hindbrain (Bingham et al., 2001, fig. 7.3) Experiments on the ectopic expression of both shh and twhh show similar results. The brain of injected embryos develops with an absence of ventricles and with no constriction at the midbrain-hindbrain boundary (Ekker et al., 1995). The notochord, floorplate and somites appear to be normal, apart from a shortening of the body axis and a curly-down tail.

7.4.11. Summary - a two-step model of zebrafish floorplate induction

Studies on zebrafish embryos mutant for members of the Nodal and Hedgehog signalling pathways have led to the proposal that the development of the floorplate in zebrafish is a two-step process. Activation of the Nodal signalling pathway controls formation of medial floorplate cells and has been shown to induce expression of Hedgehog proteins cells autonomously (Müller et
Figure 7.3. Effect of *hedgehog* gene removal on branchiomotor neuron (BMN) induction. Dorsal views of 36 hour embryos with anterior to the left. Pictures are adapted from Bingham et al., 2001. Blue staining shows the expression of *islet-1* in the CNV and CNVII. White arrowheads in all figures show the anterior and posterior limits of the CNVII, the black arrows in panel (C) highlight cells missing in the CNVII. Removal of one *hh* gene on its own causes mild to severe disruption to the induction of cranial branchiomotor neurons (panels (B) and (C)) as compared to WT (panel (A)). However, removal of both *hh* ligands causes a complete loss of branchiomotor neurons. This suggests that the removal of a single gene will be compensated for by the other *hedgehog* homologue. *echidna hedgehog*, the third zebrafish *hedgehog* homologue appears to have less of an important role in BMN induction (pictures not shown). Legend: WT, wild-type; *twhh*, tiggywinkle hedgehog; syu, sonic you mutant (which lacks *shh* activity); MO, morpholino.
7. Characterisation of monorail, a zebrafish midline mutant

et al., 2000). However, analysis of cyc mutants at late developmental stages have shown that from the end of somitogenesis onwards, at a time when cyc is no longer expressed in the body axis, MFP are seen to be formed in the posterior ventral neural tube. put this up above – not really summary It is possible that during early development the expression levels of Hh are not high enough to induce formation of MFP cells and that the induction event is instead mediated by Nodal pathway protein activity. Acting through the Cyclops signal transducers Smad2 and FAST1, a constitutively active form of Cyclops protein has been shown to initiate shh transcription (Müller et al., 2000). The Hh pathway acts to induce the formation of lateral floorplate cells (Odenthal et al., 2000). Analysis of Hh pathway mutants including syu and smu (Schauerte et al., 1998, Varga et al., 2001) shows that the medial floorplate is still formed whereas lateral floorplate cells are absent. Hh family proteins secreted from the floorplate then act to pattern the ventral neural tube (Briscoe et al., 2001, fig. 7.4). Signalling in the Hedgehog pathway is also necessary to maintain the floorplate during development. Analysis of smoothened mutant embryos (Varga et al., 2001, Chen et al., 2001) shows that in the absence of Hh activity the medial floorplate forms "islands" along the A-P axis from 27 hours of development onwards.

7.5. Other zebrafish floorplate mutants

There is a final group of zebrafish midline mutants that have not been well characterised. The mutated genes are not known and furthermore, it is not yet apparent as to whether the mutation will be in either the Hh or nodal pathway. This final group, which includes monorail, show either an indistinct (monorail and schmalhans) or irregular (iguana) floorplate. The most studied fish thus far is iguana (igu).

7.5.1. iguana

igu embryos have an irregular floorplate, abnormal retinotectal projections and turned in eyes (Brand et al., 1996). They belong to the same class of
Figure 7.4. Cartoon depicting the patterning activity of Shh in the neural tube. Shh secreted from the floorplate ("F") induces five different ventral motorneuron subtypes depending on the concentration of Shh perceived. Motorneurons induced nearer to the floorplate require higher concentrations of Shh. Shh acts by inducing the expression of transcription factors in discrete progenitor domains. These genes are thought to cross-repress each other and so sharpen up the boundary of the progenitor domain. Following domain formation, the expression of several neuronal identity genes will be induced to specify the identity of the motorneuron formed. Please refer to the text for a more complete explanation. Legend: V1, V1 neuron; V2, V2 neuron; V3, V3 neuron; MN, somatic motorneuron; D, dorsal neurons. Cartoon adapted from Briscoe and Ericson, 1999.
7. Characterisation of monorail, a zebrafish midline mutant

retinotectal mutants as *yot* and so have optic projections extending to the ipsilateral side of the tectum (class 1, see Karlstrom *et al.*, 1996). *igu* embryos also show the ubiquitous curly down tail and somite patterning abnormalities. The MFP is normal and the LFP absent, as would be predicted in a Hh pathway mutant.

7.5.2. *schmalhans*

*schmalhans* (*smh*) shows an indistinct floorplate and curly down tail (Brand *et al.*, 1996). There is no more data on this mutant available at present.

7.5.3. *monorail*

The characterisation of *monorail* (*mol*) will be discussed in the results section of this chapter.

7.6. *Function of the floorplate during development*

One of the earliest events in the wiring of the central nervous system is the formation of commissural connections, allowing information to be passed from one side of the body to the other. The extension and guidance of commissural axons starts soon after neural tube closure. Axon guidance is a specialised form of cell movement, where only the growth cone is motile. The growth cone contains cell adhesion/guidance molecule receptors that enable it to interact with its environment in response to a series of attractive and repulsive guidance cues. Axons grow in two main orientations: either longitudinally along the anterior-posterior axis, or circumferentially towards or away from the floorplate. The floorplate has a critical role in patterning both axonal projections and cell fates in the ventral neural tube during development. As new neurons are born, they project growth cones which have to be guided across the midline of the embryo, making sure that each axon reaches its appropriate target. Failure of the floorplate, roofplate and notochord to release the necessary guidance molecules will lead to neurons "missing" their intermediate or final targets. The floorplate both secretes long-distance guidance molecules and
7. Characterisation of monorail, a zebrafish midline mutant

contains contact-mediated signals in discrete cellular location to ensure correct formation of commissural projections in the embryonic spinal cord (Kaprielian et al., Colamarino and Goodman, 1995). Some of these molecules act as attractive cues and others act to repel the developing axons. These two signal types act in concert to "pull" and "push" axonal growth cones towards their destination. Guidance of axons is thought to occur as a series of small sequential steps and so understanding the complex process of axon guidance is reduced to determining the molecules involved in each of these steps (Tessier-Lavigne and Goodman, 1996).

The location and function of all the molecules that have been shown to be involved in axon guidance in the CNS ventral midline is complicated and can only be described in brief detail here. For comprehensive reviews of the subject please refer to Kaprielian et al (2001), Tessier-Lavigne and Goodman (1996) and Chisholm and Tessier-Lavigne, (1999). As far as possible, the descriptions of midline guidance functions will be based on observations in vertebrate development as this will be most pertinent to the situation in the zebrafish floorplate.

7.6.1. Long distance guidance cues

The earliest "pioneer axons" of the developing commissural projection neurons grow along the pial edges of the spinal cord until close to the floorplate. The majority of these axons then enter and cross the floorplate before turning orthogonally and extending for a variable distances (Bovolenta and Dodd, 1990). In rats, the axons only turn rostrally, whereas in other species such as chick and zebrafish this turn can be either rostral or caudal (Colamarino and Tessier-Lavigne, 1995). A second, later developing population of axons grows circumferentially towards the floorplate, but turn before crossing into the ventral midline and form ipsilateral axon tracts. Analysis of mutants such as the Danforth short-tail mouse which lacks the floorplate shows that developing axons either fail to cross into the midline, or alternatively cross the floorplate but
7. Characterisation of monorail, a zebrafish midline mutant

fail to turn into the longitudinal plane (Bovolenta and Dodd, 1991). Conversely, in mice with a mutation in Gli2, a downstream gene of the Hedgehog pathway, axons enter the midline and fail to leave again. In this mutant a selective loss of floorplate and ventral intermediate cells is seen, and disorganised axon tracts are formed in the floorplate area (Matise et al., 1999). Research has also shown that a zebrafish mutant, you -too, which shows defects in the patterning of the ventral forebrain, has a mutation in Gli2 (Karlstrom et al., 1996). The molecules involved in both long and short range guidance will be discussed next.

7.6.2. Netrin

The main long-distance chemoattractant that has been found in the spinal cord is Netrin-1. Netrin-1 is a member of a laminin-like family of proteins and is distantly related to both the Caenorhabditis elegans gene UNC-6 and more distantly to the laminin family of genes (Ishii et al., 1992, Wadsworth et al., 1996). Netrin family proteins have a remarkable degree of conservation of function across species, and in all species Netrins can function as both attractive and repulsive guidance cues (Chisholm and Tessier-Lavigne, 1999). The attractive properties of Netrin are mediated by a family of receptor proteins that are expressed in the growth cone of axons known as the DCC (Deleted in Colorectal Cancer) family (Keino-Masu et al., 1996, Chisholm and Tessier-Lavigne, 1999). Conversely, repulsive properties of Netrin are thought to be mediated by proteins of the UNC-5 family (named after the C.elegans gene UNC-5). In some cases, UNC-5 and DCC proteins have to act together to form a repulsive guidance cue receptor (Hong et al., 1999).

In Netrin-1 knock out mice, axons still show attraction to the floorplate, suggesting that molecules other than Netrin may also be involved in this process. Netrin-1 is expressed in a dorso-ventral gradient with higher expression levels ventrally, which fits in with the model of Netrin-1 acting as a long distance chemoattractant (Matise et al., 1999). Short distance contact-mediated functions have also been attributed to Netrins. For example, Netrin-1
has been found to be repellent to developing trochlear motor neurons. This suggests that the trochlear neurons may be interacting with the Netrin-1 in a different manner and is also an example of the complexity of axon guidance (Colamarino and Tessier-Lavigne, 1995). Molecules can have both attractive and repellent roles depending on the context.

Studies of retinal neuron outgrowth provides a good example of Netrin proteins acting both as an attractive and repulsive guidance cue (Höpker et al., 1999). Retinal growth cones grown on a variety of culture mediums show attractive turning responses in the presence of a gradient of Netrin-1, mediated by the receptor DCC. However, in the presence of laminin-1, the amount of cAMP in the vicinity of the growth cone decreases and the guidance cue changes from being attractive to being repulsive (Höpker et al., 1999). A gradient of cAMP across the growth cone may act to control the turning of the growth cone during axon guidance.

7.6.3. Bone morphogenetic protein 7

The roofplate of the spinal cord is a small triangular-shaped group of primary glial cells that has been shown to act as a barrier to axon growth in the developing nervous system (Steindler, 1993). Recent studies have shown the roofplate to have specific repellent activity for commissural axons (Augsburger et al., 1999). Bone morphogenetic protein 6 (BMP6), BMP7 and Gdf7 (a member of a BMP subfamily) have all been shown to be expressed in the roofplate, but only BMP7 was seen to repel growing commissural axons in explant assays (Augsburger et al., 1999). The chemorepellent activity of BMP secreted from the roofplate can be seen as "pushing" axons away from the dorsal spinal cord and so acts in concert with the chemoattractant function of the floorplate.

7.6.4. Short-range guidance cues
7. Characterisation of monorail, a zebrafish midline mutant

Having successfully reached the floorplate, developing axons are presented with two different choices; to cross or not to cross the midline. Once on the contralateral side, axons in normal developing spinal cords never cross back into the floorplate again. Analysis of Danforth short-tail mice (Bovolenta and Dodd, 1991) and mice Gli2 mutants (Matise et al., 1999) where axons either fail to cross into the midline or to turn longitudinally on the contralateral side suggests that midline crossing is dependant on contact-mediated guidance cues. Molecules in the floorplate act together to both attract axons into the floorplate, make sure that they do indeed cross the midline and finally that they leave the floorplate and form a neat fascicle on the contralateral side (Burstyn-Cohen et al., 1999). Only a small minority of developing axons stay on the ipsilateral side of the spinal cord (fig. 7.5, see Simpson et al., 2000).

7.6.5. Axonin-1, NrCAM and NgCAM

Axonin-1, NrCAM and NgCAM are all members of the immunoglobulin (Ig) superfamily of cell adhesion molecules. Axonin-1 is expressed on growth cones of growing nerves and NrCAM is expressed in cells of the floorplate (Stoeckli and Landmesser, 1995). NrCAM is permissive to axon growth and so allows axons to cross the floorplate. NgCAM, on the other hand, is expressed in longitudinal tracts either side of the floorplate and is the guidance molecule used by ipsilaterally growing neurons (Stoeckli and Landmesser, 1995). On reaching the floorplate the commissural axons have two choices - to cross the midline on an NrCAM lined tract, or to turn and grow longitudinally along an NgCAM pathway. The NgCAM is only chosen when the NrCAM pathway is unavailable (Fitzli et al., 2000), suggesting that Axonin-1 and NrCAM together play a more active role in axon guidance than NgCAM.

7.6.6. F-Spondin

The function of F-Spondin genes is not well characterised, though they have the potential to play a key role in midline signalling. F-spondin activity is needed to attract axons that have already crossed the midline back towards to
Fig. 7.5. Three cartoons depicting axon guidance by the floorplate during development. For descriptions of the guidance molecules involved please refer to the introduction to chapter 7. A full legend is included on the next page.
7. Characterisation of monorail, a zebrafish midline mutant

the contralateral floorplate boundary. Mouse embryos lacking F-spondin function show a lack of neat fascicles on the contralateral side. The receptor binding to F-spondin has not yet been identified. The formation of fascicles such as the medial longitudinal fasciculus is thought to be specified by a balance between two opposing forces: Slit protein in the midline repels axons from the midline and F-spondin activation attracts axons back to the floorplate boundary to form neat fascicles (Burstyn-Cohen et al., 1999). The zebrafish has two F-spondin genes as compared to other species; F-spondin 1 and F-Spondin 2. Two other related genes, Mindin-1 and Mindin-2 have also been found. These are more closely related to Drosophila M-spondin genes than to Drosophila F-spondin (Higashijima et al., 1997). All four genes are expressed in the embryonic zebrafish medial floorplate.

7.6.7. Altered responsiveness of growth cones

One of the main questions that studies of axon guidance have raised is how an axon can be attracted into the floorplate to cross the midline, yet does not cross back into the floorplate from the contralateral side. As the floorplate is essentially a bilateral structure, with bilaterally expressed guidance molecules (Colamarino and Tessier-Lavigne, 1995, Stoeckli and Landmesser, 1995) the axon will encounter the same attractive cues on either side of the midline. The mechanism which is used to circumvent this problem is known as altered responsiveness. As a growth cone enters the floorplate, the composition of the molecules expressed on its surface changes and it consequently becomes responsive to a different set of guidance molecules. These molecules cause the growth cone to turn orthogonally, prohibit it from re-entering the midline territory and cause it to form neat axon fascicles (Kaprielian et al., 2001).

There has been striking evidence that this mechanism is indeed used in developing growth cones. One example of this is seen in rat spinal cord, where axons on the contralateral side of the floorplate express TAG-1 but not L1 (both members of the IgCAM family). However, on the contralateral side of the
7. Characterisation of monorail, a zebrafish midline mutant

Midline L1 is expressed in commissural neurons but TAG-1 is no longer present (Imondi et al., 2000, Bovolenta and Dodd, 1991). Gli2 knock out mice, which fail to form floorplate cells, express TAG1 on both sides of the midline in contrast to the wild-type situation in which no TAG1 is expressed on the contralateral side (Matise et al., 1999). Another example of plasticity in the molecules expressed in the growth cone is the expression level of NrCAM in neurons. On the ipsilateral and contralateral sides of the floorplate, commissural axons express low levels of NrCAM. Whilst inside the floorplate territory however, NrCAM expression is upregulated to a high level (Matise et al., 1999, Stoeckli and Landmesser, 1995). In Gli2 knock-out mice, NrCAM is expressed at the same level both within and either side of the floorplate territory. Axons that do reach the midline are seen to cluster in the floorplate and turn either rostrally or caudally, failing to form fascicles. It is important to note that in knock-out animals such as the mouse Gli2 knock out, some axons do succeed in crossing the midline and turning longitudinally. Whilst the ventral midline cells of the floorplate are important for axon guidance, other cell types are also involved in this process (Matise et al., 1999).

7.6.8. Semaphorins

Altered contact mediated responsiveness has also been observed for members of the semaphorin family. Semaphorins are a group of molecules that have been found to have axon guidance properties in a wide range of species. The first family member, Sema-1a, was found as a protein antigen that directs the outgrowth of axons in the grasshopper (Kolodkin et al., 1992); Sema-2a and Sema-3a were found soon afterwards in Drosophila and chick respectively (Luo et al., 1993, Matthes et al., 1995). The receptor family for the semaphorins are the Plexins. Most semaphorins cannot bind to Plexin on their own, but instead form a complex with neuropilin1 or neuropilin2 and then bind Plexin. Other semaphorins can bind directly to Plexin (Klostermann et al., 1998). Recent research has shown that axons on the contralateral side of the floorplate are repelled by Slit-2, SEMA 3B and SEMA 3F in the mouse spinal cord (Zou et al.,
7. Characterisation of monorail, a zebrafish midline mutant

2000). Axons on the ipsilateral side are completely unresponsive to these inhibitory cues.

Four semaphorin homologues have been found in the zebrafish to date. semaphorin Z1a, semaphorin Z1b and Sema Z8 are all homologous to other known class 3 semaphorins (Halloran et al., 1998, Shoji et al., 1998, Roos et al., 1999). Sema Z7 is a class 4 homologue (Halloran et al., 1998). Analysis of these genes during development shows large areas of expression in the spinal cord and brain. All the genes have dynamic expression patterns; later on in development the expression is restricted to the fin buds and pharyngeal arches. The function of zebrafish semaphorins Z1a and Z1b have been described (Shoji et al., 1998, Roos et al., 1999). Z1a causes collapse of of the lateral line ganglion axons in vivo when applied directly to the growth cone. The motility of the growth cone recovered following collapse, which suggests that Z1a can control the morphology of the growth cone but may not affect its ability to extend (Shoji et al., 1998). Overexpression of Z1b gave rise to embryos with abnormal ventral motor axons (VMs) in the spinal cord. Most of the VMs in injected embryos exited the spinal cord but failed to extend normally into the somite (Roos et al., 1999).

It seems likely that further work will reveal more zebrafish members of the semaphorin family and the conservation of function between species for other guidance molecules suggests that they may act as repulsive signals in the floorplate.

7.6.9. Ephrins and Eph receptors

Ephrins and their receptors Eph (erythropoietin-producing haptahelical) receptor tyrosine kinases (often shortened to Eph-RTK) are another large family of molecules that mediate repulsive axon guidance in the ventral midline. Eph-RTKs can be subdivided into two subclasses (EphA and EphB) based on sequence identity and ligand specificity. ephrins, the ligands for these
receptors are also subdivided into two subfamilies; ephrin-A ligands are GPI linked to the cell membrane and ephrin-Bs are attached to the cell surface by transmembrane domains (Chisholm and Tessier-Lavigne, 1999). Generally, EphA receptors only interact with ephrin-A ligands and EphB receptors only interact with ephrin-B ligands, although several exceptions to this role exist (Imondi et al., 2000). There is substantial evidence that Eph receptors and ephrins can function as bidirectional signalling molecules. Binding of the ligand to the receptor is thought to cause phosphorylation of the ligand and lead to transduction of a signal into the ephrin expressing cell ("reverse signalling") as well as the Eph receptor cell ("forward signalling", see Chisholm and Tessier-Lavigne, 1999 for review). Class B ephrins are good candidates for being involved in contact-mediated guidance cues in the midline as they are tightly bound to the cell membrane by transmembrane domains. Three ephrins are expressed in the lateral edge of the mouse floorplate during development: ephrin-B1, ephrin-B2 and ephrin-B3 (Imondi et al., 2000). In growth cones of commissural axons that have yet to cross the floorplate, the receptor EphB1 is only expressed at low levels, whereas following decussation to the contralateral side of the floorplate, expression levels of EphB1 are increased. It is thought that working in concert, EphB1 and the three ephrin-Bs define a repulsive barrier to axon growth and so help form a neat fascicle of axons on the contralateral side of the floorplate (Imondi et al., 2000).

7.6.10. roundabout and slit

The final group of floorplate signalling molecules that I shall describe here includes roundabout (robo) and slit, genes that were first identified in the ventral nerve cord of Drosophila. There are three fish homologues of the Drosophila gene robo, called robo 1, robo 2 and robo 3 respectively (Challa et al., 2001, Lee et al., 2001). In all species studied, robo and slit have been found to have extremely similar functions. robo genes act in a similar fashion, to repel axons from recrossing into the midline. In fish, the expression patterns of all three robo genes are seen to be similar, with high expression levels in the developing
7. Characterisation of monorail, a zebrafish midline mutant

nervous system as well as in non-neural tissue such as fins and somites. The timing and pattern of the expression of these genes suggest that the fish genes may also have a role in the control of axon guidance and migration. Research on astray, one of the axon pathfinding mutants from the first Tübingen screen, has found that astray mutants have a mutation in the gene coding for fish robo2 (Fricke et al., 2001). astray mutant embryos show defects in the pathfinding of the retinal axons, which are seen misproject to the ipsilateral tectum and to several extratectal targets (Fricke et al., 2001, Karlstrom et al., 1996).

robo is the receptor for a protein found in the midline of developing embryos called slit (Brose et al., 1999, Kidd et al., 1999). Much of the work on this evolutionarily conserved sub-family of the immunoglobulin superfamily has been done in the fly (see Simpson et al., 2000). Mutations in either the Slit protein or the Robo receptor cause different phenotypes: in robo mutants axons fail to exit the midline and recross many times, whilst in slit mutants a fusion of longitudinal axon tracts is seen in the ventral midline (Simpson et al., 2000, Battye et al., 1999, Rothberg et al., 1990). In Slit mutants, commissural axons again fail to leave the midline. In the wild-type situation, the repulsive activities of Slit and Robo are needed to form axon tracts that descend on the contralateral side of the midline. Interestingly, mutants in which all three robo homologues have been removed show the same phenotype as that of a slit mutant, confirming that Robo is indeed the receptor for Slit (Simpson et al., 2000). As well as making sure that the midline is crossed and not re-entered, Robo proteins have been shown to determine the lateral position of the fasciculus formed on the contralateral side (Simpson et al., 2000). Removal of one of the three robo homologues will change the fasciculus that a developing axon will enter after crossing the midline.

The careful balance between the activities of these different yet complementary guidance molecules allows the floorplate to successfully pattern the axons of the ventral neural tube during development. The story is not
7. Characterisation of monorail, a zebrafish midline mutant

complete and there are still a large number of questions that need to be answered before the process will be fully understood.

7.7. Mapping of novel mutants using SSLPs

One of the main reasons for studying the phenotype of zebrafish mutants is to gain further insight into the functions of genes during normal development. As well as characterising the phenotype of mutants, it is possible to pinpoint the underlying mutated gene by using one of several mapping strategies. In order to find the mutated gene that underlies the phenotype of monorail, the mutant was mapped using simple sequence length polymorphic (SSLP) markers.

SSLPs are CA repeats that differ in length (i.e., are polymorphic) between different zebrafish strains. WIK and Tübingen fish are well characterised and have been used to generate a set of primers that amplify these CA repeats. Following PCR reactions with these primers, if the mutated gene of interest and an SSLP marker are linked then no recombination will occur and a single band will be seen. However, if the SSLP and the mutated gene are weakly linked, then recombination will occur and two bands will be seen that differ in size by a few base pairs. If no linkage occurs, then a random assortment of single bands and pairs of bands will be obtained. Analysis of up to 2,000 single embryos allows the linkage to an SSLP marker to be assessed. If linkage has occurred then the majority of PCR reactions on mutant embryos will produce single bands and only a few will produce pairs of bands. The number of reactions producing pairs of bands can be counted, to work out the distance between the SSLP marker and the mutated gene of interest. If from a pool of 50 mutant embryos (i.e., 100 haploid genomes or 100 meioses) only one PCR reaction produces a recombination event, then the distance between gene and SSLP will be 1 centiMorgan. A centiMorgan is defined as the number of recombination events in 100 meioses, and in zebrafish this is thought to correspond to around 625kb of DNA (Postlethwait et al., 1994).
7. Characterisation of monorail, a zebrafish midline mutant

7.8. Results

7.8.1. Phenotype of monorail

*motorail* (mol) was identified in the first Tübingen mutant screen as having a curly down tail and subtle floorplate defects (Brand et al., 1996). Subsequent observations of live embryos found that mol mutants also have an increased amount of tissue at the ventral end of the tail (fig. 7.6). *motorail* only shows relatively subtle defects, so in order to better characterise it, embryos were stained with a combination of antibodies and *in situ* hybridisation probes to analyse the midline defects.

7.8.2. Mapping of the mutated gene encoding motorail

In a parallel project another lab member, Maryam Mangoli, has been mapping the gene that encodes the mutation in *motorail*.

An F1 mapping cross was generated by crossing a heterozygous mol mutant fish in the Tübingen background with a wild-type WIK fish. The F1 generation was heterozygous for the mutation; the mutant allele came from the Tübingen background and the WT allele from the WIK background. Incrosses of F1 fish produced an F2 generation, one quarter of which would be homozygous mutants and so displayed the mol phenotype.

The gene encoding *motorail* was primary mapped by Robert Geisler (Max-Planck Institute für Entwicklungsbiologie, Tübingen) along with other mutants from Tübingen screen 1. Rough mapping placed the gene on zebrafish linkage group 17 (LG17). Subsequent mapping was performed using a simple sequence length polymorphism (SSLP) mapping strategy (Knapik et al., 1996). In order to fine map the mutation in *motorail*, pools of 48 mutant and 48 wild-type embryos were collected and used in PCR reactions to amplify SSLPs, using a set of primers that were obtained from Research Genetics. Fine mapping placed the mutated gene at a distance of less than 0.5cM from the
Fig. 7.6. Live pictures of wild-type (A and C) and *monorail* (*mol*, B and D) embryos at around 24 hours of development. *mol* has a severe curly-down tail (B) and extra ventral tail tissue (D). Arrows point to the extra tissue in the ventral tail of *mol*. 
7. Characterisation of monorail, a zebrafish midline mutant

SSLP marker Z22674 (ie one recombination event was found in reactions from 100 mutant embryos) and 30.9cM from the centromere. Comparison of the position of marker Z22674 to genes on the MGH panel suggested that a good candidate for mol would be foxA1 (see diagram in appendix 2).

In order to both confirm this result and to look for a base pair change in the coding region of the mutant foxA1 gene, PCR primers were designed that would amplify the whole open reading frame of the gene. foxA1 has an open reading frame of 1275 base pairs, which codes for a protein which is 415 amino acids long. The coding sequence was amplified from genomic DNA of single mutant and WT sibling embryos to look for the mutation underlying the mol phenotype. The gene has been cloned from both WT and mutant embryos and is currently being sequenced. Primer sequences and PCR conditions are included in appendix 1 of this thesis. Map data and sequence data are included in appendix 2.

7.8.3. Width of the floorplate in the midbrain and hindbrain

Most of the descriptions of the width of the zebrafish floorplate describe the MPF as being one cell diameter wide and the LFP as being three cell diameters wide (Odenthal et al., 2000). Whilst this is true for the floorplate of the spinal cord, in the midbrain and hindbrain the floorplate is wider; MFP marker genes including shh, twhh and foxa1 are expressed in a three cell wide band. LFP markers such as foxa2 and foxa are expressed in a band of cells six cell diameters wide. This widening of the floorplate first appears at the place where the spinal cord and hindbrain meet. In this first section of the results, the expression of floorplate marker genes is compared in mutant and sibling embryos in order to analyse floorplate development in monorail. All of the markers described in this results section are expressed in similar patterns in the spinal cord of mutant and sibling embryos. Because of this in the majority of the sections here, the description of these markers will be restricted to the midbrain and hindbrain.
7. Characterisation of monorail, a zebrafish midline mutant

7.8.4. Expression of foxa1 in mol embryos

At early developmental stages such as 50% epiboly, foxa1 is expressed in a similar way in both mol and wild-type (Fig. 7.7A, 7.7B). Expression is seen in the involuting mesendoderm that will later form the notochord and floorplate. At 24 hours of development, expression is similar in the forebrain and zona limitans intrathalamica (ZLI) of both WT and mol embryos. However, foxa1 expression in the basal plate of the midbrain and the floorplate is reduced in mutants (7.7C - 7.7F, see arrows). The expression in the MFP is present as islands of expression along the A-P axis (7.7G and 7.7H) that are three cell diameters wide. The patches of expression first appear between the 2 somite and the 22 somite developmental stage (data not shown) and are seen in stereotyped positions along the axis. Before the formation of these islands, the expression is continuous along the CNS. The positions of these islands can be confirmed by their relative proximity to the otic vesicle. These observations suggest that induction of the medial floorplate is normal but the expression of floorplate genes is not maintained during development.

7.8.5. Medial floorplate is induced but not maintained in mol embryos

The expression of foxa1 in mol embryos during development suggests that although the MFP is induced normally, it is not maintained during development. If this suggestion is correct, then analysis of other MFP markers in wild-type and mutant embryos during development will show a similar expression pattern. The initial expression of the genes will be normal, with islands of expression being formed later on in the development of mol embryos. Early expression of medial floorplate marker genes confirms this hypothesis. Analysis of the expression pattern of foxa1, shh and twhh in 50% epiboly stage embryos shows that in both WT and mol, expression is seen in the involuting mesendoderm and in the caudal end of the prechordal plate (Fig. 7.8A - 7.8F). As it is not possible to genotype mol and WT embryos at such an early stage of development, the staining was performed on a large clutch of embryos.
Figure 7.7 legend

Expression pattern of *foxa1* in *mol* and WT sibling embryos during development. Panel (A) is a lateral view and panel (B) is a dorsal view of a 50% epiboly embryo. At these stages it is not possible to separate WT from mutant embryos, but staining is uniform in all embryos suggesting that the early expression of *foxa1* is not reduced in *mol*. At later stages (Panels C - H) expression is seen to be reduced in the hindbrain and in the medial floorplate of mutant embryos as compared to siblings. Panels (C) - (F) are lateral views with anterior to the left. Arrows in (C), (D), (E) and (F) highlight areas in which expression is reduced in *mol*. These areas appear to localise to the ventral end of the midbrain-hindbrain boundary. Dorsal views at 3d (panels (G) and (H), anterior to left) show the islands of floorplate in *mol* mutants along the anterior-posterior axis (highlighted by arrowheads). These islands are visible from 24 hours of development onwards and suggest that the medial floorplate is induced, but not maintained during development.
Figure 7.7.

A  50% ep. B

foxa1

C  24h D

foxa1 WT

E  36h F

foxa1 WT

G  3d H

foxa1 WT

mol
Expression of medial floorplate markers in mol and WT embryos. 

*foxa1* (A, D, G, J, M and P), *shh* (B, E, H, K, N and Q) and *twhh* (C, F, I, L, O and R) are induced normally in mol embryos, but expression of these genes is not maintained during development. Please refer to the picture for stage, gene and genotype markings. Panels A - F show embryos at 50% epiboly. As it is impossible to separate WT and mutant embryos at this stage, the staining was carried out on a large batch of mixed mutant and sibling embryos. A - C are dorsal views of embryos and D - F are lateral views. All embryos showed a similar staining pattern suggesting that the early expression of these genes is normal. Later on in development, from 22 somites onwards, the expression of these genes is decreased. Panels G - L are dorsal views of 36 hour embryos with anterior to the left. All three medial floorplate marker genes show islands of expression along the A-P axis. The islands of expression appear to be at similar places along the A-P axis. Panels M - R show lateral views of 36 hour embryos with anterior to the left. Expression of *foxa1*, *shh* and *twhh* are all seen to be reduced in the hindbrain and the floorplate along the A-P axis.
Figure 7.8.
embryos showed a similar staining pattern. Later on in development, the expression of foxa1, shh and twhh are reduced in the MFP of the midbrain and hindbrain. In dorsal views of WT embryos, expression of these three genes is seen as a three cell wide band in the midbrain and hindbrain (7.8G - 7.8I). In mol embryos the expression is seen to be restricted to islands of expression along the A-P axis which are also three cells wide (arrows in 7.8J - 7.8L). The islands of expression appear to be in similar positions along the A-P axis as judged by the position of the otic vesicle. Lateral views of all markers confirm that the floorplate is reduced and that there is a reduction of staining in the basal plate of the midbrain (arrowheads in 7.8M - 7.8R).

7.8.6. Lateral floorplate is not formed in mol embryos

In order to study lateral floorplate (LFP) formation in mol, the expression patterns of foxa2 and foxa were analysed. Both foxa (previously known as forkhead 4) and foxa2 (axial) mark both the cells of the medial and lateral floorplate and are expressed in a band of cells 6 cell diameters wide. In WT embryos, both foxa2 and foxa are seen as bands of expression 6 to 7 cells wide in the MFP and LFP (arrowheads in 7.9C and 7.9G). Conversely, in mol embryos expression of both genes is restricted to islands of floorplate that are only three cell diameters wide (arrowheads, 7.9D and 7.9H). Lateral views comparing mol and sibling embryos show that in a similar manner to the MFP marker genes, expression of foxa2 and foxa is absent in the midbrain floorplate and the basal plate of the midbrain (arrows in 7.8A, 7.8B, 7.8E and 7.8F). Higher magnification views of foxa expression in WT and mol (see black bars in 7.9I and 7.9J) show the reduction of floorplate width in mutant embryos as compared to siblings. The islands of floorplate expression appear to be in similar positions to the islands of MFP marker gene expression seen in mol embryos. This can be judged by comparison to the position of the otic vesicle.

7.8.7. mol embryos show a reduction of gene expression in the basal plate of the midbrain as well as in the midbrain floorplate
Figure 7.9 legend

Expression of lateral floorplate marker genes in mol and WT sibling embryos. Panels A - D show expression of foxa2 and panels E - J show expression of fhk2 (also called fkd4). Panels A, B, E and F are lateral views with anterior to the left and all other panels show dorsal views with anterior to the left. Expression of both foxa2 and fhk2 show that in mol mutant embryos the medial floorplate is present in islands along the A-P axis, instead of being expressed in a wide stripe of medial and lateral floorplate cells. Arrow in A, B, E and F show an area at the ventral end of the MHB which lacks expression of both genes in mol embryos. Arrowheads in C, D, G and H highlight the reduction of staining in the midline of mol embryos. Expression appears to be thinner and only present in patches. Panels I and J are dorsal views of mol and sibling embryos stained with an in situ hybridisation for fhk2. The black bars in these panels indicate the loss of fhk2 in the lateral floorplate of mol embryos.
Figure 7.9.

A, B, C, D: foxa2 expression at 36h, with WT and mol genotypes.

E, F, G, H: fkh2 expression at 24h and 36h, with WT and mol genotypes.

I, J: Consistency of fkh2 expression at 36h with WT and mol genotypes.

Legend:
- Black arrows indicate expression changes.
- WT denotes wild type.
- mol denotes a mutated or knockdown condition.

Scale bars for reference.
7. Characterisation of monorail, a zebrafish midline mutant

Pictures show that the expression of foxa2, shh, twhh and foxa1 in the midbrain is reduced in mol embryos (fig. 7.10). Expression in the telencephalon, zona limitans intrathalamica (ZLI) and more anterior midbrain expression of all four genes is similar in both WT and mutant. Arrowheads in panels A - H highlight the reduction of expression in the basal plate of the midbrain. foxa2, shh, twhh and foxa1 all appear to be absent in the same areas in mol brains. Higher magnification pictures of the expression of foxa1 in the midbrain (20X magnification) show that in mol embryos the expression is limited to discrete patches in the basal plate of the midbrain and in the midbrain floorplate (7.10I and 7.10J).

7.8.8. Loss of the floorplate in mol causes bilateral patterning defects in the midline

One of the main functions of the floorplate in the developing midline is to guide extending growth cones across the midline. Secretion of attractive and repulsive cues from the floorplate is important for the correct formation of an early axon scaffold. In order to look at the expression of islet-1 expressing neurons in monorail, a mol -Islet1 GFP line was generated by crossing a heterozygous mol carrier with a homozygous Islet1 GFP fish (Higashijima et al., 2000). The islet1 promoter/enhancer is used to drive expression of GFP, which can be detected as a fluorescent green product when viewed under ultraviolet light. The GFP is first detected at about the 10 somite stage of development. Islet-1 marks the developing cell bodies of neurons in the nervous system (Higashijima et al., 2000, Ericson et al., 1992). The Islet -1 GFP line shows a reduced amount of expression when compared to staining with an anti-islet-1 antibody (fig. 7.11). In WT embryos the facial cranial nerve nuclei (CNVII) usually form two lines either side of the floorplate (7.11A). However, in mutant embryos the CNVII fuses to form a single nucleus (7.11B). Conversely the trigeminal cranial motor nuclei form two bilateral nuclei either side of the midline in both mol and WT (for annotation please refer to 7.11C and 7.11D). Double labelling with the anti-GFP antibody and an in situ against foxa1 shows that the
7. Characterisation of monorail, a zebrafish midline mutant

Figure 7.10 legend

Expression of foxa2, shh, twhh and foxa1 in the brain of mol and WT sibling embryos. All panels show lateral views with anterior to the left and dorsal to the top. Expression of all four genes is reduced in the hindbrain of mol embryos. Panels A and B show foxa2 expression at 36 hours and panels C and D show shh expression at 36 hours. Panels E and F show twhh expression at 3 days and panels G and H show foxa1 expression at 3 days. All genes show similar expression patterns at all developmental stages and so these pictures have been selected to show a representation of the expression of these genes during development. In panels A - H the arrowhead highlights the reduced expression in the hindbrain of mol embryos as compared to siblings. I and J show a higher magnification view of an embryo with in situ hybridisation staining for foxa1. The floorplate and hindbrain expression of twhh and foxa1 can be seen to be reduced in both mutant embryos.
Figure 7.11 legend

Loss of the floorplate in mutants causes bilateral patterning defects. All panels show dorsal views with anterior to the left. Panels A - D are anti-GFP antibody staining in a mol Islet1-GFP transgenic line. Panels E and F show double labelling with anti-GFP antibody marking Islet1 protein in brown and a foxa1 in situ hybridisation in blue. Panels G - J are anti-acetylated tubulin antibody staining on mol and siblings. Loss of the the floorplate in mol embryos causes midline patterning defects. Structures which are normally bilateral fuse in the midline. Panel B shows that in mol embryos the CNVII fuses medially but the CNV (refer to labels in panel C) still from two separate loci either side of the floorplate. Double staining with a foxa1 in situ probe shows that the fusion of motornuclei in the midline is coincident with a loss of foxa1 signal (E an F). Staining with an anti-acetylated tubulin antibody shows that the MLF does not fuse in the midline but remains either side of the floorplate. However, some growth cones can be seen to have entered the territory of the floorplate (arrowheads in I and J).
7. Characterisation of monorail, a zebrafish midline mutant

collapse of the nuclei into the midline coincides with the absence of foxa1 expression in the floorplate (7.11E and 7.11F). In some photographs, the number of CNVII nuclei appeared to be reduced in mutants as compared to WT. Analysis of 10 specimens each of WT and mutant embryos showed that on average mol had a 7% increase in the number of CNVII nuclei in the hindbrain. This percentage probably does not constitute a large enough difference in number to be significant. Staining with an anti-acetylated tubulin antibody also shows a disruption of patterning in the midline of the hindbrain (7.11G - 7.11H). In mutant embryos (7.11H and 7.11J) the medial longitudinal fasciculus (MLF) is contracted to the midline as compared to WT siblings. High magnification pictures show that some growth cones have entered the floorplate territory in the midline instead of remaining tightly bound together in the MLF (arrowheads in 7.11I and 7.11H). Tubulin staining in mol and sibling embryos suggest that in the spinal cord the motorneurons are induced normally (data not shown). The number and position of motorneurons appears to be comparable in mutant and WT embryos.

7.8.9. Other markers show defects in mol embryos

gata3 expression is seen in nuclei throughout the brain and spinal cord from early somitogenesis stages onwards (Neave et al., 1995). In mol embryos expression of gata3 is contracted to the midline as compared to expression in WT embryos (7.12A - 7.12D). Expression of gata3 at the caudal end of the ZLI is also contracted to the midline in mol embryos (7.12A and 7.12B).

col2a1 marks differentiating cells in the floorplate. In the spinal cord of mol embryos, expression of col2a1 is seen to be severely reduced as compared to WT siblings (7.12E and 7.12F). Expression in the hindbrain is normal (data not shown). The floorplate cells in the spinal cord of mol are still formed - they are recognisable by their cuboidal morphology.
Figure 7.12 legend

In situ hybridisation staining of gata3 (A - D) and col2a1 (E and F) staining on zebrafish embryos. Panels G and H show backfills of mutant and WT sibling embryos using lysinated rhodamine dextran. gata3 staining marks neuronal bodies throughout the spinal cord and brain. In mutant embryos, the two parallel lines of neuronal nuclei are seen to be contracted towards the midline. This phenomenon is highlighted by arrowheads in A - D. col2a1 marks differentiating neurons in the floorplate of the spinal cord. In mutant embryos at 24 hours the cuboidal morphology of floorplate cells is clear, but the cell are seen to be not differentiating as col2a1 staining is reduced. Panels G and H show that in mutant embryos the nucleus of the medial longitudinal fasciculus (marked by white arrows) is also contracted towards the midline. This is only faintly marked in WT due to the preparation but is clearly seen in mol.
7. Characterisation of monorail, a zebrafish midline mutant

Reticulospinal neurons in the hindbrain form the reticulospinal tract - a descending activating tract. In zebrafish the reticulospinal neurons are likely to be involved in either the initiation of swimming following an escape response, or in the maintenance of swimming (Ritter et al., 2001). In order to check for the presence or absence of reticulospinal neurons in mol, 4 day embryos were backfilled with lysinated rhodamine dextran (LRD). LRD was applied to the spinal cord with a tungsten wire and was allowed to track up the cord for 30 minutes prior to fixation. Fixed embryos were dissected and the brains were mounted between coverslips to allow visualisation using a confocal microscope (Fig. 7.12G and 7.12H). Analysis of both WT (7.12G) and mutant (7.12H) embryos shows that the reticulospinal neurons and mauthner neurons are present in mol. However, the nucleus of the medial longitudinal fasciculus (nMLF) is present, but is formed in a more medial position than in WT siblings (7.12H). This nucleus is found in a position very close to that of the CNIII which has already been shown to be absent in mol embryos (refer to Fig. 7.11). This suggests that the loss of the CNIII nucleus is a result of a failure of induction of this specific cell type, rather than a general mis-patterning of this area of the midbrain.

7.8.10. mol embryos have a reduction of specific neurons in the midbrain and isthmic region of the brain

The enzyme Trytophan hydroxylase A has an important function in the formation of serotonin (5HT) in the serotoninergic nuclei of zebrafish (fig. 7.13). The gene ZFTPHa marks serotoninergic nuclei throughout the brain of zebrafish, from around 35 hours of development onwards (H. Teraoka, pers. comm.). Analysis of TPHA in mol mutant embryos shows that expression is reduced in the anterior part of the raphé nucleus (arrowhead in 7.13B). Dorsal views of the same embryos show that TPHA expression is present in the pineal organ of both WT and mutant even though the raphé nucleus shows a severe reduction of expression (7.13C and 7.13D). Double staining with foxa1 in blue
Figure 7.13 legend

*mol* embryos show a loss of neuronal nuclei in the midbrain and hindbrain. Panels A - D show TPHα staining which marks sertotoninergic nuclei of the raphé nucleus. *mol* embryos show a severe loss of the expression of this marker gene as compared to siblings (A - D, arrow highlights absence of expression in *mol*). A small patch of expression remains at the caudal end of the raphé nucleus. Expression in the pineal organ is seen to be similar in both mutant and siblings. Double labelling with *foxa1* and an anti-GFP antibody which detects Islet1 protein in *mol* Islet1-GFP fish shows that *mol* mutant embryos lack the CNIII nucleus (arrowheads, panels E and F). This is coincident with a reduction of *foxa1* expression in this area in *mol* embryos.
Figure 7.13.

A  3d  B  3d
TPHa  WT  TPHa  mol

C  3d  D  3d
TPHa  WT  TPHa  mol

E  36h  F  36h
Islet1 and foxa1  WT  Islet1 and foxa1  mol
7. Characterisation of monorail, a zebrafish midline mutant

and anti-GFP antibody in the Islet1 GFP line shows that mol embryos also lack oculomotor cranial motor nucleus (CNIII). In WT embryos, the CNIII is clearly present in two bilateral loci either side of the midline (7.13E, arrowheads). However in mol embryos, as well as a reduction of foxa1 expression in the area of the basal plate of the midbrain floorplate there is a loss of CNIII motor nuclei (7.13F, arrowheads).

7.8.11. Endodermal genes are expressed normally in mol embryos

Many of the genes expressed in the embryonic midline during development are also expressed in the endoderm (fig. 7.14). Furthermore, HNF3α knock-out mice (HNF3α is the mouse homologue of foxa1) show liver defects and die of an inability to metabolise food (Kaestner et al., 1999). There are no embryonic defects reported in the HNF3α knock-out mouse. Expression of foxa1 in fish is also seen in the developing endoderm from early somitogenesis stages onwards. Expression in mol embryos is similar in both sibling and mutant (36 hours, 7.14C and 7.14D). At three days of development, expression can be clearly seen in the liver and the gut tube (7.14E and 7.14F). Expression is the same in both WT and mutants. Other genes, including shh, twhh and axl are also expressed in the early developing endoderm. The expression of all these genes is the same in both WT and mutant (data not shown).

7.8.12. foxa1 acts downstream of two signalling pathways

In order to study the function of foxa1 during development, and to analyse genes that are active upstream of foxa1, in situ hybridisations were performed on zebrafish mutants known to have defects in signalling in either the Nodal or Hedgehog pathways. maternal zygotic one-eyed-pinhead (mzoep) mutant embryos (Carmany-Rampey and Schier, 2001) lack all foxa1 expression (Fig. 7.15A and 7.15B) suggesting that foxa1 acts downstream of the Nodal signalling pathway. In situ hybridisations on smoothened (smu) embryos, show
Figure 7.14 legend

Endodermal expression of foxa1 is normal in mol embryos as compared to WT siblings. All panels are dorsal views with anterior to the left. At 36 hours of development, expression of foxa1 can be seen as an area of staining between the caudal limit of the eye and the anterior limit of the spinal cord. Higher magnification views of staining show that expression is similar in both mol and siblings (C and D). At 3 days of development, expression can be seen in the liver and also in the gut tube (E and F). There does not appear to be a difference in expression between mutant and WT.
7. Characterisation of monorail, a zebrafish midline mutant

Figure 7.15. legend

*In situ* hybridisation expression of *foxA1* in *mzoep* and *smoothened* mutant embryos. Panels (A) to (D) are lateral views with anterior to the left and dorsal to the top. Panels (E) and (F) are dorsal views with anterior to the left. *foxA1* expression is completely absent from *mzoep* embryos (7.15B) as compared to WT siblings (7.15A). However, expression of *foxA1* was seen in the midline of *smu* mutant embryos although expression in the brain was reduced (7.15D and 7.15F). Arrowheads in 7.15C and 7.15D highlight the reduction of *foxA1* expression in *smu* mutant embryos. Arrows in 7.15E and 7.15F show the same reduction of expression pattern from a dorsal view. These results suggest that during midline development, *foxA1* acts downstream of the Nodal pathway. During brain development, *foxA1* acts downstream of the Hedgehog pathway, as in *smu* mutants which lack nearly all Hedgehog signalling expression is only absent in the midbrain and hindbrain.
7. Characterisation of monorail, a zebrafish midline mutant

expression of *foxa1* in the midline was normal, (7.15C - 7.15F) but the expression in the mutant brain was severely reduced (7.15D). A small amount of expression remained in the basal midbrain. *smoothened* mutants have been shown to lack nearly all Hedgehog function (Varga et al., 2001, Chen et al., 2001). Therefore, during development *foxa1* acts downstream of these two signalling pathways in different tissues.

7.8.13. Expression of *shh* and *twhh* in *smoothened* mutants

If the expression of *foxa1* is indeed acting downstream of the Hedgehog pathway in the brain, why is there reduced expression of *twhh* and *shh* in the brain of *mol* mutants? The most parsimonious explanation for this result is that there is a feedback loop in the Hedgehog pathway, whereby expression of *foxa1* is necessary for maintenance of *hh* expression in the brain during development. *In situ* hybridisation staining on *smu* mutant embryos (Fig 7.16) shows that expression of *shh* and *twhh* is reduced in the brain as compared to WT (7.16A and 7.16C, WT embryos; 7.16B and 7.16D smu). This expression pattern is similar to that seen in *mol*. Therefore the reduction may indeed be due to failure of maintenance of Hedgehog signalling in the brain during development as a result of a loss of *foxa1* expression. Figure 7.17 is a diagram that summarises the activity of *foxa1* in the midline during development.

7.9. Discussion

7.9.1. Phenotypic summary

*mol* embryos induce the expression of MFP marker genes during early development, but do not maintain their expression in the floorplate. From 22 somites onwards islands of expression are seen at stereotyped positions along the A-P axis. The LFP is not formed in *mol* embryos. Expression of *foxa1, shh, twhh, foxa2* and *foxa* in the ZLI and the basal plate of the midbrain is seen to be reduced in mutants as compared to WT siblings. *monorail* embryos also lack the induction of a few specific neuronal subtypes in the midbrain and hindbrain.
7. Characterisation of monorail, a zebrafish midline mutant

Figure 7.16.

In situ hybridisation staining on smoothened mutant embryos at 36 hours using probes for shh (7.16A to 7.16D) and twhh (7.16E to 7.16H). All panels are lateral views with anterior to the left and dorsal to the top. shh is seen to expressed in the same areas in both smu mutants (7.16B and 7.16D) as WT siblings (7.16A and 7.16C). However, expression of twhh is seen to be the same in the midline of smu mutant (7.16F) but reduced in the brain of smu (7.16H) as compared to WT siblings (7.16G). This expression pattern mirrors that see in monorail mutant embryos and provides further evidence that expression foxA1 may be necessary for the maintenance of twhh expression in the brain during development. Arrowheads in (G) and (H) highlight the area of brain which lacks foxA1 expression in mutants (H) but is present in WT (G).
Figure 7.16.

A

shh

B

36h

shh

C

36h

shh

D

WT

shh

E

36h

twhh

F

WT

twhh

G

36h

twhh

H

WT

twhh

smu

smu

smu
Figure 7.17. Diagram summarising a possible genetic pathway for the function of *foxA1* during development. Expression of *foxA1* in the ventral midline acts downstream of the Nodal signalling pathway. Expression of *foxA1* in the brain acts downstream of the Hedgehog signalling pathway and is necessary for the maintenance of *twhh* expression in the brain. *shh*, however, does not appear to need *foxA1* expression for its maintenance as expression *shh* in the brain of both *monorail* and *smoothened* mutants is seen to be normal. In this diagram, question marks denote areas of the pathway where other genes may be involved that have not yet been identified.
7. Characterisation of monorail, a zebrafish midline mutant

In areas where the floorplate expression of foxa1 is absent, nuclei are either seen to be closer to the midline than normal or to be fused in the midline. monorail embryos have a curly down tail, extra ventral tail tissue and a patchy floorplate. The curly-down tail phenotype is variable, with some crosses producing embryos with less severe curly down tails. The curly down tail phenotype is seen in many mutants with midline defects (Brand et al., 1996), although it is not yet understood why it occurs. During development, the cells of the notochord vacuolise and this may force the developing tail to straighten. For some reason in Nodal and Hedgehog pathway mutants this straightening does not occur. It may be possible that these pathways signal to cause vacuolisation of notochord cells. The mis-shaping may also be linked to the extra tissue seen in the ventral tail extension. It is possible that a mild ventralisation occurs as a result of impaired midline signalling and this affects the amount of tissue seen in the ventral tail. In ventralised embryos, a mis-specification of the dorsal-ventral axis early on in development causes extra tissue to form ventral cell types at the expense of dorsal tissue.

Simple sequence length polymorphism mapping has identified foxa1 as a potential candidate for the mutated gene in monorail. In situ hybridisation analysis has shown that floorplate cells have patchy expression along the A-P axis of every MFP gene tested thus far. Analysis of genes including foxa and foxa2 that mark both the MFP and the LFP have shown that the lateral floorplate is absent in mol.

The fusion of several cranial motorneuron nuclei in the midline of the embryo may be due to a structural absence of floorplate cells, or to a change in the activity of the floorplate cells. In areas where foxa1 is still present these nuclei are still found in the correct places either side of the floorplate. Three distinct types of neuronal cell bodies are also seen to be absent in mol embryos: serotoninergic neurons of the raphe nucleus, nuclei of the oculomotor (CNIII)
7. Characterisation of monorail, a zebrafish midline mutant

motornucleus and a set of small nuclei thought to be part of the anterior trigeminal motor nucleus (CNV).

7.9.2. Region specific maintenance of the floorplate during development

Early expression of genes including foxa1, shh and twhh appears to be the same in both mutant and sibling embryos. However, as development proceeds this expression is not maintained and islands of expression are seen along the length of the A-P axis. During development, mol embryos are seen to express all floorplate genes in stereotypical positions along the A-P axis. The A-P position of the islands can be assessed in two ways - by the relative position of the otic vesicle and by double labelling with an antibody against Islet1 protein. This suggests that there is a control mechanism that acts to maintain the expression of these markers at defined positions. For example, foxa1, shh, twhh, foxa and foxa2 are all seen to be present in the area in which the CNV develops, but absent from the anlage of the CNVII. It is not easy to predict how this mechanism may be acting. It may be possible that there are as yet unidentified upstream genes in the floorplate that are region specific and are not effected by the mutation in foxa1. Alternatively, another member of the fox family of genes may control the maintenance of floorplate in these areas.

7.9.3. Migration and specification of neuronal number is intact in monorail

CNVII neurons are unusual in zebrafish, in that they are induced in rhombomere 4 and then migrate caudally during development, finally occupying positions in rhombomeres 6 and 7 (Chandrasekhar et al., 1997). Studies of the migration of facial branchiomotor nuclei in mouse (Garel et al., 2000) show that a combination of environmental cues and cell autonomous mechanisms are needed for the correct selection of migratory pathway by CNVII neurons during development.

Analysis of the anti-GFP antibody in mol-Islet1 GFP fish suggests that, although in mutant embryos the CNVII neurons are found in a more medial
7. Characterisation of monorail, a zebrafish midline mutant

position than in WT siblings, the caudal migration of these neurons is intact. If the caudal migration was disrupted then the nuclei of the CNVII would be seen to occupy more rostral positions either side of the midline in mol embryos. Other mutants, including the zebrafish gastrulation mutant trilobite, have been seen to have defects in this tangential migration (Bingham et al., 2002).

In the majority of mol embryos stained with anti-GFP antibody, the number of Islet1 expressing neurons was comparable in both wild-type and mutant. In some mol embryos, the number of CNVII neurons was seen to be reduced, but analysis of a large number of specimens found this reduction to be small and was probably not a significant phenotype.

Other cranial motornuclei are seen to be present in the normal position in mol embryos. The CNI (olfactory nucleus), CNIV (trochlear nucleus, data not shown) and CNV (trigeminal nucleus) motornuclei are present as bilateral areas of expression either side of the floorplate. However, the CNIII (oculomotor nucleus) is absent in mutant embryos.

In mouse, members of the winged-helix family of transcription factors (which corresponds to the forkhead box domain family in zebrafish) have been implicated in the direct control of neuronal nuclei number during development. Thymocyte winged helix (TWH) is a forkhead domain transcription factor that is expressed in mouse spinal cord neurons and interneurons (Dou et al., 1997). Removal of the function of the gene TWH during development causes a reduction in the number of spinal cord neurons, although the identity and positions of the neurons was not affected. It is not yet known whether monorail encodes a null mutation or a hypomorphic mutation. Injection of foxa1 sense RNA into WT embryos would allow the effect of overexpression on the number of CNVII nuclei in the brain to be further investigated.

7.9.4. Tubulin staining shows subtle defects in mol embryos
7. Characterisation of monorail, a zebrafish midline mutant

Staining with an anti-acetylated tubulin antibody shows that the medial longitudinal fasciculus (MLF) is seen to contract slightly towards the midline in the hindbrain area adjacent to the otic vesicle. Some axons are seen to enter the floorplate territory instead of remaining tightly bound to the MLF. The defects in the tubulin staining pattern seen in this area appear to be more subtle than the fusion of motornuclei seen with Islet1 antibody labelling. One possible reason for this apparent discrepancy may be that the MLF is formed at an earlier stage of development than the CNVII. At the earlier developmental time point, the floorplate cells are present and so the MLF is formed in the correct position. However, later on in development the floorplate is not maintained and so the CNVII nuclei are able to enter the midline territory.

Interestingly, cyclops mutant embryos also have impaired pathfinding and fasciculation of axons in the spinal cord and brain (Hatta et al., 1992). Ventral neurons in the anterior brain of cyc mutants appear to be reduced in number, whereas the primary and secondary motorneurons in the spinal cord appear to be unaffected (Hatta et al., 1992). It is likely that the similarity in axonal pathfinding and induction of motorneurons in both monorail and cyclops mutant embryos is due to a failure of floorplate formation or function in both mutants.

7.9.5. Induction of 5HT and CNIII nuclei in the midbrain

mol embryos are seen to lack two nuclei in the isthmic region and the midbrain - the serotoninergic nuclei of the raphé nucleus and the nucleus of the third cranial nerve. There are two possibilities that could account for the absence of these nuclei. Either the mutation in foxa1 directly effects the induction of the nuclei, or the absence of raphé nuclei and oculomotor nuclei is due to a reduction of twhh and shh activity in the brain of mol embryos. As foxa1, shh and twhh all show a reduction of expression in these areas it is not possible to predict which of these two possibilities are correct. Injection of hedgehog RNA into mol embryos to try and rescue the phenotype would allow
7. Characterisation of monorail, a zebrafish midline mutant

this question to be analysed. If foxa1 acts via hh genes to induce these nuclei, then this induction would be cell non-autonomous.

7.9.6. hedgehog family genes induce branchiomotor neurons at discrete locations along the anterior-posterior axis

Overexpression of shh and twhh sense RNA in cyclops mutant embryos has been shown to restore the numbers of branchiomotor neurons (Chandrasekhar et al., 1998). Similarly, in sonic-you (syu) embryos the numbers of branchiomotor and spinal motor neurons are reduced, but are never completely absent (Schauerte et al., 1998). shh and twhh function as partially redundant signals to induce motorneurons during zebrafish development. Analysis of different alleles of syu has shown that CNV neurons need the highest levels of shh for their induction. CNVII neurons were variably affected by a loss of shh and even in the strongest allele studied a few CNVII neurons were present (Chandrasekhar et al., 1998). Interestingly, no loss of neurons anterior of the CNV neuron was reported in syu mutants and it is suggested that the induction of these neurons may require another hedgehog family member (Schauerte et al., 1998, Chandrasekhar et al., 1998). Other studies have shown that downstream targets of shh also have region specific roles. For example, detour, encodes a Hh pathway target genes that is required for Hedgehog mediated events in the hindbrain but not the spinal cord (Chandrasekhar et al., 1999).

These results are in good agreement with the model whereby a loss of twhh and shh in the brain causes a lack of induction of serotoninergic nuclei and CNIII nuclei. To test this hypothesis, future work on this project will include injections of twhh, shh and foxa1 into wild-type embryos to try and increase the number of these neurons in the brain. Injections of twhh, shh and foxa1 into mutant embryos should rescue the loss of these neurons which is seen in monorail. Injections of shh sense RNA into wild-type fish has already been seen to cause an increase in the number of ZFTPHA expressing neurons (H. Teraoka,
7. Characterisation of monorail, a zebrafish midline mutant

pers. comm.). As both *shh* and *twhh* are thought to signal through the same receptor complex on the cell membrane, an overexpression of either gene will cause an increase in total Hedgehog protein in injected embryos. This in turn may cause an upregulation in the amount of foxa1 RNA and consequently cause an increase in the levels of *shh* and *twhh* RNA that may act to increase the number of ZFTPHα positive cells.

7.9.7. fox gene function in other species

Studies on the mouse homologue of foxa1, HNF3α, have highlighted the important role of this gene in the function of endodermally derived organs including the liver (Kaestner *et al.*, 1999). HNF3α knock-out mice are born at the same weight as littermates, but rapidly lose weight 10 to 14 days after birth. Studies of hormones involved in glucose homeostasis have shown that HNF3α is an essential activator of the glucagon gene *in vivo*. Even though the knock-out mice had food in their stomach, the absence of the HNF3α gene meant that they died of starvation (Kaestner *et al.*, 1999). In *Caenorhabditis elegans* the HNF3a homologue *pha-4* has been shown to be important in regulation of endodermal organogenesis. In the absence of the gene, there was a failure of initiation of transcription of a large number of downstream endodermal genes. Interestingly, mutations in the DNA binding domain of *pha-4* caused a change in the timing of transcriptional activation rather than in the activation itself. In this project I have focussed on the neural aspects of the *mol* phenotype. Analysis of genes that are expressed in the endoderm suggests that early endoderm formation is intact in mutant embryos. However, the HNF3α knock-out mice also appear to have normally developed endodermal structures and only have defects in liver metabolism. It would be interesting to use look at genes that would test endodermal organ metabolism in monorail embryos.

7.9.8. Partial redundancy of foxa2 function in mol embryos may underlie the ventral neural tube patterning defects
7. Characterisation of monorail, a zebrafish midline mutant

*foxa2* (previously known as *axial*, *forkhead1* and *HNF3β*) is another type 1 forkhead domain transcription factor that is expressed in the midbrain, CNS midline and endoderm during development (Odenthal et al., 1998, Strähle et al., 1996). In a similar manner to the data I have presented on *foxa1*, *foxa2* is activated by both the Nodal and Hedgehog signalling pathways. At the end of gastrulation, *foxa2* expression is induced in the neuroectoderm that overlies the mesendoderm in which it is initially expressed. By 24 hours of development, the expression is localised to the ZLI, basal midbrain and both the MFP and LFP. The expression pattern of both *foxa2* and *shh* are very similar in the MFP and midbrain / hindbrain. Overexpression studies using both *shh* and *foxa2* suggest that the regulation of these two genes is interdependent (Strähle et al., 1996).

It is possible that some of the midline defects in *mol* embryos are due to impaired *foxa2* signalling. It has been suggested that the absence of *foxa2* expression in the ventral neural keel of *cyc* embryos is responsible for fusion of the ventrolateral axon tracts and nuclei (Hatta et al., 1991). Therefore, the reduced expression of *foxa2* downstream of *foxa1* in *mol* mutant embryos may also be the cause of the ventral neural tube mis-patterning that is observed.

7.10. Conclusions

In this chapter, the characterisation of a novel zebrafish midline mutant *monorail* provides new evidence for the role of *forkhead box* domain transcription factors during ventral midline patterning. *foxa1* acts downstream of both the Nodal and Hedgehog signalling pathways to insure correct patterning of neurons found on either side of the floorplate and to induce the formation of CNIII and serotoninergic neurons in the brain. As part of the Hedgehog signalling pathway, expression of *foxa1* is necessary to maintain the expression of *shh* and *twhh* in the brain during development. As expression of *foxa1* is active downstream of the *hedgehog* genes in the brain, the most likely explanation for this regulation of *hedgehog* function is that *foxa1* mediates a
7. Characterisation of monorail, a zebrafish midline mutant

feedback loop in the Hedgehog pathway to maintain expression of both shh and twhh.

7.10.1. Future experiments

Work on monorail has suggested several future experiments that should be done. Rescues of the monorail phenotype are underway at the time of writing this thesis. Injection of foxa1 sense RNA into mol embryos may rescue the phenotype. It will particularly interesting to assay the position of the CNVII neurons in the hindbrain and the presence of absense of serotonergic neurons in the isthmic region of the brain. Rescues will also be done using twhh and shh sense RNA to see if injection of these genes can rescue some of the mol phenotypes that I consider to be due to failure of correct signalling in the Hedgehog pathway.

Injection of the foxa1 morpholino into WT embryos looks promising and should be followed up with studies looking at specific markers following the generation of knock-out embryos.

There are several other midline marker genes whose expression I have still to analyse in monorail. These include the f-spondin and mindin genes, cylcops, floatinghead and phox2a / souless. Double staining with both in situ hybridisation probes and antibodies will allow some of the characterisation to be repeated in more detail. Finally, I have some mol/syu double mutant fish which are nearly old enough to be genotyped and will allow me to study the function of mol in the absence of shh signalling.
8. General Discussion

CHAPTER EIGHT

General Discussion

Discussions of the experimental results and their relationship to data in other species have been included at length in the results chapters of this thesis, therefore the general discussion given here will be restricted to the following topics.

8.1. Part 1: The role of P2 receptor genes during development

The aim of the first part of this thesis was to investigate the role of P2 receptor signalling during vertebrate development. Evidence from studies in chick, *Xenopus* and rat embryos (Meyer *et al.*, 1999, Ryten *et al.*, 2001, Cheung and Burnstock, 2002, Burnstock 2001b, Bogdanov *et al.*, 1997) had already shown that P2 receptors may indeed have an important function in controlling developmental processes. Furthermore, the ability of this receptor family to regulate developmentally important processes, including cell proliferation, differentiation and death (Burnstock, 2001b), has been demonstrated in a number of studies. An elevation in the level of internal calcium ions has also been documented in a number of embryonic cell types in response to extracellular nucleotides (Nakaoka and Yamashita, 1995, Qi *et al.*, 2000).

In the first part of this thesis, I present evidence of three novel P2 receptor genes that are expressed during zebrafish development. Sequences from all three genes were acquired from the Washington University EST database and subsequent analyses have shown interesting and specific expression patterns.

8.1.1. p2x3 may be involved in mediating a sensory response to touch stimulation
The zebrafish p2x3 gene is first expressed at the 4-5 somite stage of development, as a pair of lateral stripes either side of the developing neural plate. Later on in development, the expression is found in cells of the trigeminal ganglion and Rohon-Beard cells in the developing spinal cord. Double labelling with an anti-HNK1 antibody showed p2x3 to be restricted to neural crest derived cells of the trigeminal ganglion. The trigeminal ganglion is unique among cranial ganglia, as it is derived from two different cell types. Trigeminal ganglion cells that are formed early during development are derived from placodes, thickenings of the neuroectoderm found in bilateral positions adjacent to the neural tube. Conversely, later developing trigeminal ganglion cells are derived from the neural crest, a mobile population of differentiating cells formed at the border of the neural and non-neural ectoderm. Expression of p2x3 in HNK-1 negative cells suggested that expression was restricted to late developing, and therefore neural crest derived cells. This hypothesis was supported by in situ hybridisations on narrowminded mutant embryos (Artinger et al., 1999). narrowminded mutants lack cranial neural crest cells and failed to show any p2x3 expression as compared to wild-type siblings. This expression of the zebrafish P2X3 receptor in crest derived trigeminal cells is consistent with studies in other species, in which P2X3 is seen in both small diameter cells of the trigeminal ganglion that are Vanilloid receptor-1 positive and dorsal root ganglion (DRG) cells (Guo et al., 1999). p2x3 in situ in late stage embryos did not show any expression in DRG. Following removal of Rohon-Beard cells by apoptosis, expression of p2x3 was limited to the trigeminal ganglion and lateral line ganglion. One possibility for this altered expression of the fish p2x3 parologue is that a different P2 receptor undertakes this function in DRG in fish.

8.1.2. Function of the P2X3 receptor during development

The expression of the p2x3 in sensory cells of the developing head and trunk suggests that the P2X3 receptor may have a role in an early sensory response. Rohon-Beard cells in fish form an early sensory network that is thought to allow the embryo to respond to touch. This network offers the
8. General Discussion

embryos some protection, by allowing it to respond to, and try to move away from, potentially damaging tactile stimulation. Attempts to study the function of the P2X3 receptor during development were not very successful. Patch-clamping of Rohon-Beard cells or trigeminal ganglion cells proved to be very difficult in our hands. Movies taken to show the response of embryos to different ATP concentrations in the presence of different P2 receptor blockers gave mixed conclusions. Whilst ATP application gave a clear response that was not mimicked by control application of water, P2 receptor blockers were not able to inhibit this response. There are several possibilities that could account for these observations. The ATP could be mediating a response via another receptor subtype other than a P2 receptor and so would not be affected by the antagonists. Alternatively, for some reason the antagonists might not be able to interact with the receptors whereas the ATP solution was. More experiments would have to be devised in order to study this effect.

8.1.3. Future work on p2x3

If someone were to continue the work on P2X3 receptors there are several lines of research that it would be interesting to pursue. Firstly I would try and search for possible duplications of the p2x3 gene in the genome. Searching the Sanger centre database or library screening a zebrafish cDNA library would hopefully identify any potential p2x3 orthologues.

Secondly it would be interesting to look in more depth at the films of ATP injections onto WT and antagonist incubated embryos. A first step would be to quantify the magnitude of response following agonist application. It is possible that following antagonist application, the duration of the response of the embryo to agonist stimulation would be reduced. A graticule applied to the lens of the camera would allow the size of the response to be measured. The length of response can be measured by looking at the films as freeze frames.
Finally, I believe that an antibody against the P2X₃ protein would be very useful in the further study of this receptor subtype.

8.1.3. p2x4 is expressed in the retina, gut and brain during zebrafish development

*p2x4* expression was first detected at around two and a half days of development, the time at which the gastrointestinal tract (GIT) was formed. Expression was seen in several tracts of the developing hindbrain, retina and in the GIT. Frozen sections of one month old fish larvae showed that expression was also seen in some cells of the notochord and branchial arches as well. Interestingly some blue staining was also present in the contents of the stomach. It is possible that this is due to cells that have sloughed off from the GIT wall and are now found in the stomach contents. *p2x4* expression was also seen in GIT tissue taken from a one year old fish, whereas control liver tissue did not stain blue suggesting that even in adult fish the P2X₄ receptor is active in GIT tissue. This expression pattern of *p2x4* in gut is in good agreement with studies in other species which report expression in stomach, colon and pancreas tissue. However, in other species, including rats and humans, expression is also observed in the thyroid, lung, testis, vas deferens and placenta among other places. It is possible that in fish other P2 receptor subtypes undertake this function, or that differences in tissue functions between fish and mammals means that the receptor is not needed in these tissues. The expression of *p2x4* in the zebrafish GIT suggests that it may have a function in initiating peristalsis following stretch-mediated ATP release. P2 receptors have been described as being expressed on the hollow organs of the body and may have a role in signalling following stretching of epithelia. The late expression of *p2x4* suggests that is does not have a role in controlling development of the GIT, but rather acts as a house-keeping gene that is active following GIT formation. The observation that *p2x4* expression was lacking from known zebrafish GIT mutants fits in well with this model of *p2x4* being expressed in differentiated GIT cells. In the retina and hindbrain, *p2x4* is probably functioning in synaptic
transmission or has a neuromodulatory role in these tissues. This would be in good agreement with studies in other species (Wheeler-Schilling et al., 2000).

8.1.4. p2y11 expression during zebrafish development

The expression of p2y11 during zebrafish development is localised to a ventral stripe of cells that may correspond to mesendoderm. Expression was first seen at 50% epiboly, and was then present in all developmental stages tested. In late stages, expression was restricted to anterior mesoderm and endoderm. This expression pattern is unusual and it hard to predict its function. It may be that the P2Y₁₁ receptor is functioning to control the release of Calcium ions in these tissues during development. This is the first description of a P2Y receptor during zebrafish development and is the first description of in vivo expression of p2y11 in any species.

There are two possible functions for P2 receptors during zebrafish development. Firstly, the genes may be important for specific developmental functions including cell migration, proliferation and differentiation (Burnstock, 2002). The second possible role is that these receptor subtypes show the physiological functions known to occur in these tissues during development and do not themselves influence the developmental process.

8.1.5. The zebrafish as a model to study P2 receptor gene expression during development

The zebrafish is extremely good model to use for the study of developmental processes and novel gene expression patterns. The rapid development and transparency of zebrafish embryos makes for easy elucidation of the temporal and spatial expression of novel genes. There are also several genetic techniques available that allow the genetics underlying developmental processes to be studied. A combination of fish mutant lines, morpholinos and over-expression studies have helped with the dissection of the many signalling pathways involved in fish development.
8. General Discussion

8.1.6 Future plans

The first section of this thesis provides descriptions of three novel zebrafish P2 receptor subtype genes expressed in dynamic and specific expression patterns. However, the work presented in this thesis only represents a pioneering study in what could be a very fruitful area of research. It is likely that zebrafish contain many more P2 receptor genes that have yet to be indentified and that may have important roles during development. The extra genome duplication event that it thought to have occurred in teleost fish means that there is a possibility that two or three homologues exist for any gene found in other species (see Van de Peer, 2001). It will also be interesting to compare the pharmacological properties of zebrafish P2 receptors to the properties of those found in other species. It was not within the aim of this thesis to study the pharmacological properties of P2 receptors, instead I chose to try and address the molecular biology and expression of novel P2 receptor genes during zebrafish development. One study of the zebrafish P2X3 receptor has been published (Boué-Grabot et al., 2000), and this has shown that the zebrafish homologue has unique pharmacological properties, with the fastest activation and desensitisation times of any known P2 receptor subtype. A combination of studies on the expression patterns and pharmacological properties of zebrafish P2 receptors should give insight into the function of this family of genes during fish development.

Designing assays to test the function of these P2 receptor genes proved to be difficult in the zebrafish. In order to study the function of fish homologues of these receptors, I think it will be necessary to express these receptors in xenopus oocytes, or another comparable system.

8.2. Part 2: Screening and characterisation of novel zebrafish mutants
8. General Discussion

The aim of the second part of this thesis was twofold: to screen for novel zebrafish axon guidance mutants and also to characterise a zebrafish mutant isolated in the first Tübingen zebrafish screen. Both projects have been fairly successful. The two screens which I took part in during my PhD work isolated over 500 new zebrafish mutants which are currently being re-identified and better characterised. In parallel, the characterisation of monorail has provided new insights into the patterning of the ventral midline during development.

Screening and characterisation of zebrafish mutants has already proved to be extremely successful in the isolation of genes that are important for zebrafish development. Mutants found in the first Tübingen screen have been instrumental in understanding the Hedgehog (Schauerte et al., 1998, Karlstrom et al., 1999) and Nodal pathways (Feldman et al., 1998, Gritsman et al., 1999), the formation of the floorplate (Odenthal et al., 1998), convergent extension (Heisenberg et al., 1999) and somitogenesis (Stickney et al., 2000) among other development processes.

The new brain pathfinding mutants will hopefully also be equally useful in helping find novel genes involved in the formation of the axon scaffold. The heterozygous carrier fish found in Tübingen have now been outcrossed and are being grown up at UCL. There is a lot of work to still be done testing complementation to known brain mutants and characterising and mapping these new mutants. From the 500 mutants that were found in the screen, 10 or 20 will be chosen to study in the first instance. These mutants may well be selected for having an easily recognisable and highly specific phenotype. Other may show similar phenotypes to previously characterised mutants but encode different genes involved in the same developmental process, as judged by a complementation test.

8.2.1. Monorail suggests novel roles for forkhead domain genes during development
SSLMP mapping has suggested that a good candidate for the gene mutated in monorail is foxa1. foxa1 is currently being sequenced from WT and mutant embryos to try and identify the mutation. The work on monorail has given new insight into the function of forkhead domain genes during development.

The expression of foxa1 had already been described in the literature (Odenthal et al., 1998) but no function had yet been suggested for the fish gene. Other forkhead domain genes have already been described as having roles in the development of the gut, midline and neural crest. For example, foxa2 acts to pattern the ventral neural tube (Strahle et al., 1993) and foxd3 (see Odenthal et al., 1998) is expressed in neural crest cells. However, the finding that foxa1 acts both to control patterning of the cranial motornuclei either side of the floorplate and to control the induction of CNIII and raphe nuclei in the midbrain and isthmic region of the brain presents a new role for this gene family during development.

8.2.2. Zebrafish forkhead box genes have different roles in midline patterning

The zebrafish has nine homologues of the Drosophila gene forkhead. foxa2 (also known as axial or fkd1) has been shown to be a downstream target gene of the Hedgehog pathway which is known to be necessary for the induction of floorplate cells. A loss of foxa2 expression in cyclops mutants is thought to contribute to the lack of ventral CNS midline specification in cyclops (Strähle et al., 1993). The patchy expression of foxa2 in mol suggests that foxa2 may act together with foxa1 to induce the cells of the floorplate. foxa3, fkh2, foxa1 (fkd7) and foxa2 are class 1 Forkhead domain transcription factors and are all expressed in the ventral neural tube. It will be interesting to see how these genes are related to each other. Both foxa (fkd4) and foxa3 (fkd2) have yet to be well characterised and may be found to have equally important roles in the patterning of the ventral midline during development. foxa3 expression is seen to be strong in the cells of the prechordal plate and hatching gland, and this
gene may be important for patterning of ventral tissue anterior of the floorplate (Odenthal et al., 1998). Other forkhead genes have been found to important for development of the neural crest (foxd3 / fkd6) or neural ectoderm (foxb1.2 / fkd3).

8.2.3. foxa1 and foxa2 have similar expression patterns and regulatory pathways

The expression patterns of foxa1 and foxa2 are very similar - both genes are expressed in the ZLI, midbrain and floorplate. foxa1 and foxa2 also both appear to be regulated by two pathways during development. Expression in the brain and MFP is under the control of the Nodal pathway, whereas expression in the LFP is induced by the Hedgehog pathway. Furthermore, both genes are classified as class 1 forkhead box genes (Odenthal et al., 1998). The similarities between these two genes suggest to me that the function of class 1 proteins may be highly conserved during evolution.

8.2.4. Future plans

Future work could include transplantation studies to test whether the induction of motorneurons by foxa1 is cell autonomous or not. Since foxa1 is a transcription factor it is likely that it acts cell autonomously in the ventral midline. Transplantation of WT cells into mol hosts may enable us to show that foxa1 can induce motorneurons cell non-autonomously. Analysis of mol:syu double mutants which are currently growing up to breeding age in the aquarium will allow us to study the function of foxa1 in the absence of shh. A more indepth analysis of the relationship between foxa1 and foxa2 may also help show whether these genes act together to pattern the ventral CNS, or whether one gene acts downstream of the other in the midline. Other experiments that need to completed include injections of hedgehog RNA into mol mutant embryos to try and rescue the loss of TPHa positive and CNIII neurons in the midbrain. The mutation in foxa1 needs to be indentified and it would be interesting to know if the gene encodes a null mutation or is acting as a hypomorph.


9. Bibliography


9. Bibliography


related to mammalian Oct4 and is essential for formation of the midbrain and hindbrain, and for pre-gastrula morphogenesis. *Development* **129**, 905-16.


9. Bibliography


9. Bibliography


9. Bibliography


9. Bibliography


protein necessary and sufficient for early endoderm formation in zebrafish.


9. Bibliography


9. Bibliography


9. Bibliography


9. Bibliography


**Bibliography**


9. Bibliography


9. Bibliography


Vial, C. and Evans, R. J. (2001). Smooth muscles does not have a common P2x receptor phenotype: expression, ontogeny and function of P2x1 receptors in mouse ileum, bladder and reproductive systems. *Auton Neurosci* **92**, 56-64.


9. Bibliography


Appendix One

Polymerase chain reaction

Cloning of foxA1 from wild-type and monorail

The sequence of the forward primer was
CTTCAGGAGGAGTTACCCGC
The sequence of the reverse primer was
GGGGGTAATGAGGAAGTTGGT
Primers were stored 1 at 00mM

Reaction conditions
14.2μl DEPC water
2μl buffer with Mg²⁺
1μl forward primer at 20 pmol/μl
1μl reverse primer at 20 pmol/μl
1μl template RNA from pool of 50 embryos
0.5μl dNTPs, 10mM
0.3μl Taq polymerase (Sigma)

Cycling parameters

94°C 5 minutes
35 cycles of:
94°C 1 minute
58°C 1 minute
72°C 2 minutes
72°C 10 minutes
4°C Hold
Appendix 2.1

Appendix 2.1. Open reading frame sequence of foxA1.
Gene expression pattern

Embryonic expression of a P2X3 receptor encoding gene in zebrafish

William H.J. Nortona,*, Klaus B. Rohr³, Geoffrey Burnstockb

aDepartment of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK
bAutonomic Neuroscience Institute, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, UK

Received 3 July 2000; received in revised form 6 September 2000; accepted 6 September 2000

Abstract

From studies performed primarily in mammals, it is thought that the P2X3 purinoreceptor is involved in mediating sensory and nociceptive signals in adult tissues. However, little is known concerning the expression or function of P2X family genes during early development. Here we describe the expression of a gene (p2x3) encoding a P2X3 receptor during zebrafish development. We find that zebrafish p2x3 is expressed in the anlage of the trigeminal ganglion from very early stages of development, most likely in neural crest derived trigeminal cells as opposed to placode derived cells. p2x3 is also expressed in the spinal sensory Rohon-Beard cells and in the putative posterior lateral line ganglion.

© 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: ATP gated ion channel; Rohon-Beard cells; Trigeminal; HNK-1; Posterior lateral line ganglion

1. Results

The P2X3 receptor belongs to a group of ATP gated ion channels (purinoceptors) that are characterized by two transmembrane domains with a large extracellular loop and intracellular C and N terminals. P2X3 has been extensively studied with respect to function and pharmacology in adult neural tissues (Burnstock, 2000; Garcia-Guzman et al., 1997). The adult expression of P2X3, as studied in mouse, rat, and human, is restricted to specific groups of neural cells (Bradbury et al., 1998; Eriksson et al., 1998; Xiang et al., 1998) that include the nociceptive neurones of the trigeminal and dorsal root ganglia (DRG), and a subset of axon terminals in the dorsal spinal cord. Immunohistochemical studies suggest that P2X3 expression in the trigeminal ganglion may be specific to neural crest derived neuronal subtypes (Chen et al., 1995).

Little has been done to characterize P2X genes during development and in the present study we describe the embryonic expression pattern of a gene encoding P2X3 in zebrafish. An EST clone (Genbank accession numbers A1588308 and A1588766) was obtained from the Washington University zebrafish EST project (W.U. Zebrafish Genome Research database: www.wustl.wuzgr.edu). DNA sequence analysis shows that the clone is 1724 bp long and contains an open reading frame of 416 amino acids, encoding for a putative full length cDNA. A similarity tree based on the amino acid sequences of various P2X genes confirms that the zebrafish gene encodes for a purinoceptor. The highest homology was seen to both the human and rat P2X3 receptors (Fig. 1). The pharmacological properties of this protein have recently been investigated in cell culture (Egan et al., 2000).

Expression of the zebrafish p2x3 gene starts around 6 somite stage in two lateral stripes adjacent to the dorsal neural keel and caudal to the eye (Fig. 2A,B). This position is consistent with expressing cells being cranial neural crest. A pair of bilateral spots of stronger expression at the posterior end of the stripes (between the developing eye and ear) persists throughout development (Fig. 2A,C,F,I). At around 12 somite stage (Fig. 2C–H), weak and transient expression is visible in developing cells of the rostral diencephalon and telencephalon (Fig. 2G), and in scattered cells within the spinal cord (Fig. 2E,H). Both sites of expression disappear shortly after this stage, although it is possible that either all, or a subset of the cells in the trunk are early Rohon-Beard cells.

During late somitogenesis, expression in sensory Rohon-Beard neurones in the spinal cord becomes apparent (Fig. 2I). Rohon-Beard neurones are easily recognizable due to their prominent size and position in the dorsal neural tube (Grunwald et al., 1988; Metcalfe et al., 1990). p2x3 expression at this site decreases over time consistent with apopto-
reveals that the bilateral sites of body labels developing neurones - initially trigeminal ganglion between the eye and the otic vesicle, we combined in situ hybridization with both HNK1 and Islet-1 antibody staining caudal to the ear. This is the position occupied by the posterior domains appears at the anterior border of the first somite, just 20 hpf (Fig. 3B,C; arrows), the rostral domains of expression are again to a single domain by around 30 hpf (Fig. 3A). By 50 hpf (Fig. 3B,C; arrows), the rostral domains of expression are adjacent to the eye and a second pair of bilateral expression domains appears at the anterior border of the first somite, just caudal to the ear. This is the position occupied by the posterior lateral line ganglion (Metcalfe et al., 1985) suggesting late p2x3 expression may be within sensory neurones of this cranial ganglion.

The domain of p2x3 expression between the eyes and ears elongates slightly at 20 hpf, frequently fragments into two groups of cells around 24 hpf (Fig. 2I; arrows) and coalesces again to a single domain by around 30 hpf (Fig. 3A). By 50 hpf (Fig. 3B,C; arrows), the rostral domains of expression are adjacent to the eye and a second pair of bilateral expression domains appears at the anterior border of the first somite, just caudal to the ear. This is the position occupied by the posterior lateral line ganglion (Metcalfe et al., 1985) suggesting late p2x3 expression may be within sensory neurones of this cranial ganglion.

To determine the identity of cells expressing p2x3 between the eye and the otic vesicle, we combined in situ hybridization with both HNK1 and Islet-1 antibody staining (Metcalfe et al., 1990; Korzh and Thor, 1993). HNK1 antibody labels developing neurones - initially trigeminal ganglion neurones, later Rohon-Beard cells and eventually many CNS and PNS neurones. Double staining with HNK1 reveals that the bilateral sites of p2x3 expression are cells of the trigeminal ganglion (Fig. 3D), but mostly not the differentiated HNK1 expressing neurones themselves (Fig. 3E,F).

Islet-1 immunostaining labels the nuclei of primary neurones (Korzh and Thor, 1993). Double staining with anti Islet-1 antibody shows that a subset of Islet-1 positive trigeminal cells coexpress p2x3 and this is consistent with HNK1 double staining (Fig. 3G). It is known that the trigeminal ganglion is composed of both placodal and neural crest derived cells (reviewed for chick by Noden, 1993; for mammals by Verwoed and van Oostrom, 1979). The localization of p2x3 transcripts to HNK1 negative cells is consistent with expression being restricted to the neural crest derived component of the trigeminal ganglion, as early differentiating neurones derive from the placodal lineage, whereas the neural crest give rise to later differentiating neurones and glia (Moody et al., 1989; D’Amico-Martel and Noden, 1983). The neural crest origin of p2x3 expressing trigeminal cells is also supported by the early lateral stripes of expression which are in the right place to be migratory neural crest that could include the cells that later express P2X3 in the trigeminal ganglion.

We further analyzed p2x3 expression in a zebrafish mutant, narrowminded (Artinger et al., 1999). The mutation leads to a reduced number of early neural crest cells and eliminates all Rohon-Beard neurones. Embryos (24 hpf) show little response to touch, reduced pigmentation and a reduction in the size of the trigeminal ganglion as only placode derived cells are present (Artinger et al., 1999). In situ hybridization on 24 hpf narrowminded mutants shows a lack of p2x3 expression in both the trigeminal anlage and Rohon-Beard neurones (Fig. 3H,I), confirming the identity of p2x3 expressing trigeminal cells as neural crest derivatives.

In summary, embryonic zebrafish P2X3 expression in putative neural crest derived trigeminal cells correlates well with expression in the adult trigeminal ganglia in mammals (Chen et al., 1995). Expression in Rohon-Beard neurones is consistent with a role for P2X3 in many primary sensory neurones. Rohon-Beard neurones are not present in higher vertebrates but in embryonic and larval fish and frogs these CNS cells mediate sensory responses prior to the differentiation of the dorsal root ganglia (Clarke et al., 1984).

2. Experimental procedures

In situ hybridizations were performed according to standard procedures (MacDonald et al., 1997). The HNK1 antibody (Sigma) was used as described in Metcalfe et al. (1990). Combined p2x3 and HNK1 staining was carried out sequentially with a short fixation step after the in situ hybridization. The narrowminded mutant allele is m805 (Artinger et al., 1999).
Fig. 2. Early expression of p2x3 in putative central and peripheral neural cells. Lateral (A,F,K) and dorsal (B,D,E, with anterior down) views of embryos showing p2x3 expression at the stages indicated. (A,B) Expression in dorsal cells adjacent to the midbrain and anterior hindbrain. The arrowheads indicate spots of higher expression. (C-H) Twelve somite stage (12s) embryos showing expression in putative trigeminal ganglion cells (arrowheads, C,D,F) in the forebrain (G) and in various spinal cord cells (E,H). (I) Twenty-four hour embryo in which expression in the putative trigeminal ganglia cells has condensed to two spots (arrows) and in which expression in dorsal Rohon-Beard neurons is prominent (the inset shows a dorsal view of the spinal cord). (J,K) Twenty-four hour embryos showing p2x3 expression (in blue) and Islet-1 antibody stain (in brown). Ventral brown cells are primary motorneurons (arrowhead), and dorsal black cells show double labelled Rohon-Beard cells (arrow). Abbreviations: e, eye; d, ventral diencephalon; o, otic vesicle; t, telencephalon.

Acknowledgements

We thank Nigel Holder for his inspiration that led to the initiation of this project. We are grateful to Stephen Wilson and Jon Clarke for generous support and help with the manuscript, Lukas Roth and Claire Russell for invaluable technical advice and Anthony Graham for helpful discussions. Kristin Artinger kindly supplied us with narrowminded mutant embryos. Anti Islet 1/2 antibody was kindly provided by Vladimir Korzh. This study was supported by funding from the Wellcome Trust and BBSRC to Stephen Wilson and from Roche Bioscience to G.B.
Fig. 3. $p2x3$ is expressed in trigeminal cells and in putative posterior lateral ganglion cells. Dorsal (A,B,D,G with anterior to the left) and lateral (C,H,I) views of embryos showing $p2x3$ expression (in blue) and HNK1 antibody labelling (in brown) at the stages indicated. (A–C) Expression in the trigeminal ganglia (A) and the posterior lateral line ganglia (B,C). The arrowheads indicate the trigeminal ganglia and the arrows the posterior ganglia. (D–F) Double labelling of $p2x3$ and HNK1 in the trigeminal ganglia. Arrows show the split domains of $p2x3$ expression in the trigeminal ganglia (E). (G) Double labelling of $p2x3$ and Islet-1 in the trigeminal ganglion. An Arrowhead indicates a putative placode derived cell that only expresses Islet-1 and the arrow points to a double labelled putative neural crest derived cell. (H,I) Lateral views of $p2x3$ expression in both a narrowminded mutant (H) in which no staining can be seen and a wildtype sibling (I) which shows normal expression. Abbreviations: o, otic vesicle; s, first somite.

References


