REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree PhD Year 2005 Name of Author AMOYEG M.

Rhombomere boundaries and hindbrain patterning in the zebrafish.

Marc Amoyel

Presented for the degree of Doctor of Philosophy
February 2005.

Division of Developmental Neurobiology
National Institute for Medical Research
The Ridgeway
London NW7 1AA

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

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract.

The vertebrate hindbrain is a segmented structure, divided into repeating segments called rhombomeres, at the interface of which boundary cells are induced as a specific cell type. Previous work has found that, in the zebrafish hindbrain, there is a distinctive organisation of glia and neurons along the anterior-posterior axis within each rhombomere, with specific neurons at the centre or boundary regions, separated by a glial curtain adjacent to the boundary. Using molecular markers, I have characterised the organisation of several cell types within each rhombomeres, and found that from 24 hours of development, neurogenesis occurs predominantly adjacent to the rhombomere boundaries, which suggests a role for boundaries in establishing this pattern. The aim of this work was to test a possible patterning role of boundaries, and to establish a genetic hierarchy between boundary genes by carrying out knockdowns of boundary-specific genes.

The secreted factor *wnt1* is expressed in dorsal boundaries and is a candidate mediator of a potential signalling role of boundaries. From many lines of evidence in previous work, *wnt1* was known to promote proliferation of neural progenitors and inhibit neuronal differentiation. I found that in the zebrafish hindbrain, however, knockdown of *wnt1* or of the Wnt pathway effector *tcf3b* causes expansion of boundary-specific markers and loss of neurogenesis in non-boundary regions. Knockdown of *rfng*, a component of the Notch pathway required for boundary expression of *wnt1* also causes expansion of boundary markers. Wnt1 is necessary for the expression of *ash* and *ngn1* proneural genes, and of *delta* genes, which in turn block ectopic boundary marker expression. Thus, *wnt1* mediates a lateral inhibition of boundary fate by regulating neurogenesis in hindbrain segments. Taken together with concurrent work in the lab, the model derived from this is remarkably similar to the regulatory interactions occurring at the dorso-ventral boundary of the *Drosophila* wing imaginal disc, and indicates recruitment of a conserved network of genes in non-homologous tissues.

Acknowledgements.

I would like to thank first of all my supervisor, Dave, for giving me such a great project to work on, and for providing help and support throughout, particularly for writing this thesis.

I am also grateful to everyone in the Wilkinson lab, particularly Cheng, for sharing reagents, ideas, and the hindbrain boundary project, Andrea, for teaching me everything I know about zebrafish, and Sebastian, for invaluable IT and cloning help.

Other people in the institute have given me help and advice, I hope I don't forget anyone : Qiling, Alex and lab members, Isabel from Derek's lab (for most of the probes), James and lab members, Nobue and everyone on the 6th floor, the Guillemot lab (particularly Olivier), and Ang lab members (Yannis and Bertrand) for all the fun.

This is also a good opportunity to thank my friends (David, tu l'as ta citation...), and especially my flatmates, Firas and Malte in Mecklenburgh Sq, and Hadrien in Arlington Rd. Thanks for putting up with me...

Un grand merci à toute la famille, Laure (et Steve, felicitations pour Emma), Anne (and Erik), Steph, papa, mum, papi et mimi.

And of course, Florence... Merci de m'avoir soutenu et d'avoir été là pendant tout ce temps, et d'être venue à Londres, merci.

Contents.

List of figures.

List of abbreviations.

AER : Apical ectodermal ridge.

AP : Alkaline phosphatase.

bHLH : Basic helix-loop-helix.

BMP : Bone morphogenetic protein.

DAB : 3,3-diaminobenzidine.

DIG : Digoxigenin.

DNA : Deoxyribonucleic acid.

FGF : Fibroblast growth factor.

GFP : Green fluorescent protein.

MHB : Midbrain-hindbrain boundary.

MO : Morpholino oligonucleotide.

MZ : Mantle zone.

PCP : Planar cell polarity.

PBS : Phosphate buffer saline.

PBST : Phosphate buffer saline containing 0.1% Tween-20.

r1-r7 : rhombomere 1-7.

RNA : Ribonucleic acid.

mRNA : Messenger RNA.

VZ : Ventricular zone.

ZLI : *Zona limitans intrathalamica.*

ZPA : Zone of polarising activity.

Introduction.

Segmentation in development.

A prominent feature of the embryonic development of many vertebrate and invertebrate species is the formation of repeated morphological units from an initially uniform tissue, a process known as segmentation. There are essentially two distinct types of segmentation processes. Segments, or metameres, can be generated sequentially, for instance in the case of vertebrate somitogenesis, where somites are added bilaterally at the posterior end of the embryo (reviewed by Pourquie, 2001). In the second mechanism, segments are derived from the subdivision of an initially uniform field of tissue, as occurs in segmentation of the *Drosophila* syncytium (Akam, 1987; Ingham, 1988; Scott and Carroll, 1987; St Johnston and Nusslein-Volhard, 1992).

Much of the current knowledge on the processes of segmentation comes from studies in *Drosophila* (Fig.1A, Nusslein-Volhard and Wieschaus, 1980), where several gene classes sequentially and hierarchically regulate the subdivision of the embryo into segments (reviewed in Akam, 1987; Ingham, 1988; Scott and Carroll, 1987; St Johnston and Nusslein-Volhard, 1992). Maternal and gap genes define domains of expression, leading to pair-rule gene expression in alternating stripes corresponding to future parasegments. Segment

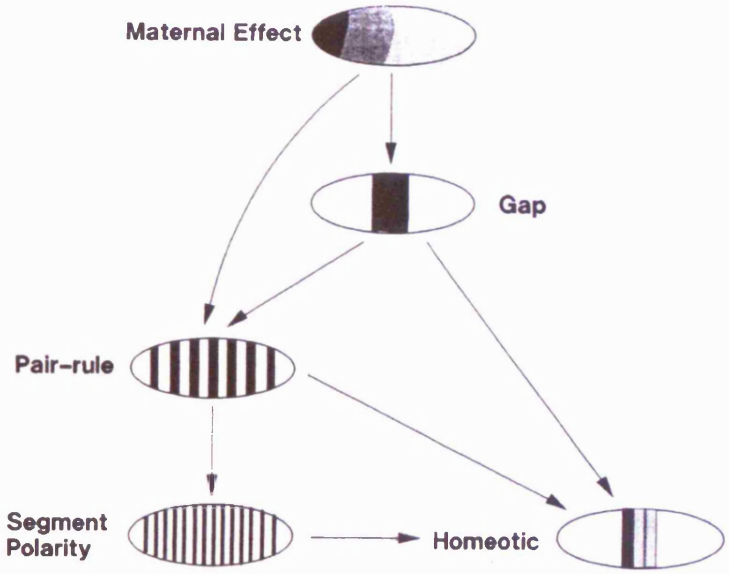
polarity genes are expressed at the anterior or posterior border of each parasegment and ensure that their interfaces are maintained, as well as contributing to the patterning of the segments. Together, these genes are known as segmentation genes, as they regulate the formation of repeated segmental units. Parallel to this process, segment identity genes act, such that each parasegment acquires its own identity, for instance distinguishing abdominal from thoracic segments. Genes of the homeotic complex (HOM-C) are responsible for determining segment identity, such that mutation or overexpression of these genes leads to homeotic changes in the fate of the segment, for example wings growing instead of halteres (Lawrence and Morata, 1994; Lewis, 1978).

Although the mode of segmentation used by *Drosophila* appears to be a specialised adaptation for its early development which occurs before cellularisation, homologues of many of the genes involved are also found in other species to have similar roles, and act in similar regulatory hierarchies (Peel, 2004; Tautz, 2004). In vertebrates, there are two obviously segmented structures: the mesoderm is divided in segments called somites, and the hindbrain, whose units are termed rhombomeres.

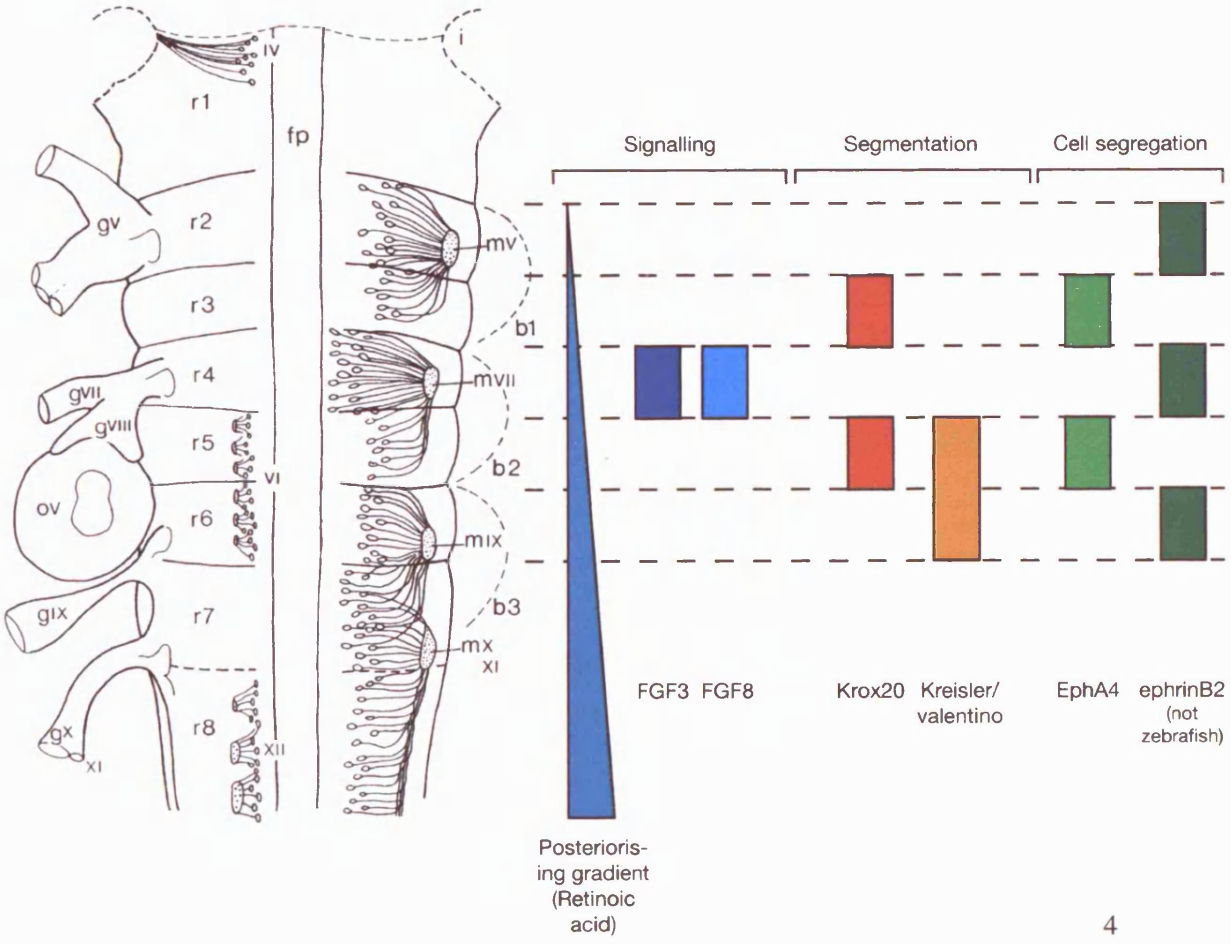
Fig. 1 : Segmentation in Drosophila and in the vertebrate hindbrain.

A : Diagram schematising segmentation in Drosophila (from Wilkinson and Krumlauf, 1990). Graded maternal factors lead to the establishment of discrete domains of gap gene expression, and together with gap genes, to pair-rule gene expression. In turn, pair-rule genes activate expression of segment polarity genes, and the combined activity of gap, pair-rule and segment polarity genes gives rise to the correct pattern of homeotic (segment identity) gene expression. B : Morphological and molecular segmentation of the vertebrate hindbrain (adapted from Lumsden, 1990). Diagram of a chick hindbrain showing the relationships between rhombomeres (r1-r8), cranial nerves (roman numerals) and branchial arches (b1-3). On the right, selected gene expression patterns are shown in relation to rhombomeres, and the posteriorising retinoid gradient is schematised. OV : otic vesicle, fp : floor plate, i : isthmus.

A


Maternal Effect
Gap
Pair-rule
Segment Polarity
Homeotic

B


Signalling
Segmentation
Cell segregation
r1
r2
r3
r4
r5
r6
r7
r8
fp
gv
gVII
gVIII
ov
gIX
gX
XI
IV
VI
XII
i
mV
mVII
mIX
mX
XI
b1
b2
b3
FGF3 FGF8
Krox20 Kreisler/ valentino
EphA4 ephrinB2 (not zebrafish)
Posterioris- ing gradient (Retinoic acid)

The vertebrate hindbrain as a model for studying segmentation.

The vertebrate hindbrain has proven to be an important model of segmentation in vertebrates. Unlike somitogenesis, which involves cyclic gene expression within a growth zone underlying the sequential formation of segments, hindbrain segments are laid down in a non-sequential manner within the neural epithelium to give rise to 7 or 8 (depending on species and nomenclature) rhombomeres.

Evidence for and functional significance of segmentation in the hindbrain.

Morphological swellings in the hindbrain were first described as early as 1823 by von Baer in chick embryos (cited by Gräper, 1913). Subsequently, work by several authors in many vertebrate species found similar swellings, and after a suggestion by Remak in 1850, Orr (1887) firmly demonstrated a relationship between what he termed "neuromeres" and cranial nerves in lizard embryos (see literature reviews by Locy, 1895; and McClure, 1891), suggesting that neuromeres constitute functionally important developmental units (an issue that was strongly debated, some believed that neuromeres resulted from mechanical pressures, while others believed their importance was secondary to that of mesoderm segmentation, see reviews by McClure, 1891; Neal, 1918). The term "rhombomere" was first used by Meek (1907), to distinguish the neuromeres of the rhombencephalon (hindbrain), with reference to seagull development. Rhombomeres and their relationships with cranial nerves, have since been reported in all classes of vertebrates, including elasmobranch (cartilaginous) and

teleost (bony) fish, reptiles, birds and mammals, including human embryos (Gilland and Baker, 1993; Meek, 1909; Meek, 1910; Streeter, 1908; Vaage, 1969). For a long time it was unclear what the significance of these rhombomeres was (see for instance Neal, 1918), but an important clue came from studies showing that segmentation of the hindbrain underlies the differentiation pattern of neurons, demonstrating that segmentation is an underlying cause of patterning in the hindbrain (Lumsden and Keynes, 1989). Motor neurons differentiate first in even-numbered segments, and even after differentiation occurs in odd-numbered segments, the motor root exit points remain located in the even-numbered rhombomeres (Fig.1B, Lumsden and Keynes, 1989). Thus, a two-segment periodicity is present in the hindbrain, warranting comparisons with *Drosophila* segmentation. Different rhombomeres give rise to motor neurons with different identities. for instance, neurons contributing to the trigeminal (Vth) nerve are located in rhombomeres 2 and 3 (r2 and r3), while neurons of the facial (VIIth) nerve are in r4 and r5 in the chick. Segmentation is also reflected in the reiteration of other classes of neurons, with some segmental differences in cell number (Clarke and Lumsden, 1993). Evidence of a functional importance for this metameric cellular organisation has been obtained from studies of the respiratory rhythm regulation (reviewed in Champagnat and Fortin, 1997). Pairs of rhombomeres contain a "rhythm generator" that is a functionally autonomous unit generating rhythmic motor activity, but require an "intersegmental co-activator" for coordination across the whole hindbrain.

Another potential functional significance of the segmentation of the hindbrain is observed in the organisation of neural crest cells, which migrate out

of the neural tube to give rise to many derivatives, including the entire facial skeleton and to connective tissue between muscles and bones, as well as peripheral nerves and glia (Noden, 1983; Noden, 1988). Neural crest streams form only adjacent to rhombomeres 2, 4 and 6 and migrate respectively into the first, second and third branchial arches (Lumsden et al., 1991). Thus, neural crest migration and motor nerve exit points are maintained in register with their respective branchial arches. Moreover, neural crest cells and their derivatives from different segmental origins are kept separate throughout development, and form sharp borders when they become juxtaposed in the structures which they contribute to, indicating that the initial segmental identity of the neural crest cells is maintained (Kontges and Lumsden, 1996). Although recent evidence shows that neural crest cells can change identity in a different environment, it is clear that hindbrain segmentation is important for segmental specification and for the proper patterning of neural crest cell migration (reviewed in Trainor and Krumlauf, 2000b; Trainor and Krumlauf, 2001).

Mechanisms of segmentation in the hindbrain.

Several genes involved in the segmentation process of the hindbrain have been identified, using different approaches in different species (Fig.1B). One of the first segmentation genes to be identified is a zinc finger transcription factor, *krox20*, which is expressed in rhombomeres 3 (r3) and 5 (r5) (Wilkinson et al., 1989a). Mouse embryos in which *krox20* has been inactivated completely lack these two rhombomeres, although the rest of the hindbrain is normally segmented (Schneider-Maunoury et al., 1997; Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993). Moreover, ectopic expression of *krox20* is sufficient

to confer odd-numbered segmental identity (Giudicelli et al., 2001) Another transcription factor, *kreisler/maf-b* (or *valentino* in zebrafish) defines the domain that will give rise to r5 and r6, and acts upstream of *krox20* in r5 (Cordes and Barsh, 1994; Frohman et al., 1993; McKay et al., 1994; Moens et al., 1998).

The regulation of the expression of these genes is not yet well understood, but recent work has established the signalling cascades leading to proper segmentation gene expression in the posterior hindbrain (Fig.1B). First, posteriorising factors involving a posterior-to-anterior gradient of retinoic acid control the initial expression of the segmentation genes (reviewed in Gavalas and Krumlauf, 2000). Subsequently, a organising centre is established in r4 (Maves et al., 2002; Walshe et al., 2002). Two fibroblast growth factors, *fgf3* and *fgf8*, are expressed in r4, and act cooperatively with retinoic acid signals to pattern r5 and r6. *vhnf1* is a target of retinoic acid signalling in these rhombomeres, and is required for the r4-derived FGF signals to induce expression of *valentino* in r5 and r6, and consequently *krox20* expression in r5 (Hernandez et al., 2004; Marin and Charnay, 2000; Wiellette and Sive, 2003). Such cascades have not been established for more anterior regions, but several other factors that are important for segmentation have been identified. For instance, *iro7* is expressed in the anterior hindbrain and has an antagonistic relationship with *vhnf1*. A sharp border between r4 and r5 is established by the mutual antagonism between these two transcription factors, but *iro7* does not appear to be required for the process of segmentation itself (Lecaudey et al., 2004).

There are no homologies between the genes regulating the subdivision of the hindbrain into rhombomeres and those controlling segmentation in *Drosophila*. However, studies in these systems have unveiled that there may be

common principles between the two processes. For instance, early in patterning, graded factors are used to establish broad domains. Another intriguing parallel is that of pair-rule expression, which can be compared to the expression of *krox20* in alternating segments. This may suggest that segmentation is most easily attained by conferring different properties to alternating segments. However, to date, very few segmentation genes have been identified in vertebrates, such that it has been difficult to establish clear genetic hierarchies.

Acquisition of segment identity.

Rhombomere identity is regulated by the Hox gene family, which are homologues of the HOM-C genes in *Drosophila*, which regulate segment identity and pattern the nervous system (reviewed in Doe and Scott, 1988; Krumlauf, 1992; McGinnis and Krumlauf, 1992; Scott and Carroll, 1987). A defining feature of Hox genes is their organisation into clusters, and the collinearity of their expression patterns with their genomic organisation (Fig.2). Hox genes are expressed in nested patterns in vertebrate embryos, with genes that are located 3' in the clusters expressed more anteriorly than genes that are at the 5' end of clusters. In the hindbrain, boundaries of Hox gene expression correspond to boundaries between rhombomeres (Wilkinson et al., 1989b), and particular combinations of Hox genes are expressed in each segment at specific levels, although no Hox genes are expressed anteriorly to r2 (Fig.2, Hunt et al., 1991; reviewed in Krumlauf, 1994; Lumsden and Krumlauf, 1996; and Moens and Prince, 2002).

Mutation or overexpression of Hox genes in mouse or chick embryos can lead to homeotic transformations, establishing Hox genes as segment identity

regulators. For instance, *hoxb1* is expressed in r4 and mutation of this gene leads to a transformation of certain r4-specific neurons into neurons characteristic of r2, while overexpression of *hoxb1* in r2 causes cells to adopt r4 fate, as seen by the migration and innervation phenotypes of the motor neurons in these rhombomeres (Bell et al., 1999; reviewed in Lumsden, 2004; Studer et al., 1996). Hindbrain-derived neural crest exhibits anterior transformations in *hoxa2* mutants (Gendron-Maguire et al., 1993; Rijli et al., 1993). However, few other dramatic homeotic transformations have been observed, and this may be due to the fact that Hox genes are expressed in overlapping patterns and can functionally substitute or synergise with each other (Davenne et al., 1999; Gavalas et al., 2003; Gavalas et al., 1998; Greer et al., 2000; Krumlauf, 1994). Work in the zebrafish embryo has shown the important roles played by Hox cofactors, Meis and Pbx proteins (reviewed in Moens and Prince, 2002). Importantly, in embryos in which most Hox function has been removed, the hindbrain appears to adopt in its entirety an r1 fate (Waskiewicz et al., 2002). This result is consistent with Hox genes not being expressed in r1, and with a model in which Hox genes act to confer a specific identity to segments. However, certain observations suggest that Hox genes may do more than regulate the identity of rhombomeres. The phenotype of *hoxa1* mutant mouse embryos, in which r5 is deleted and r4 strongly reduced, implies that *hoxa1* is a segmentation gene (Dolle et al., 1993; Mark et al., 1993). The zebrafish homologue of *hoxa1*, *hoxa1a*, is not expressed in the hindbrain (McClintock et al., 2001), but its function has been taken over by one of the *hoxb1* homologues, *hoxb1b* (McClintock et al., 2002). Thus, comparing the functions, as well as the expression patterns and genomic organisation of Hox genes across vertebrates

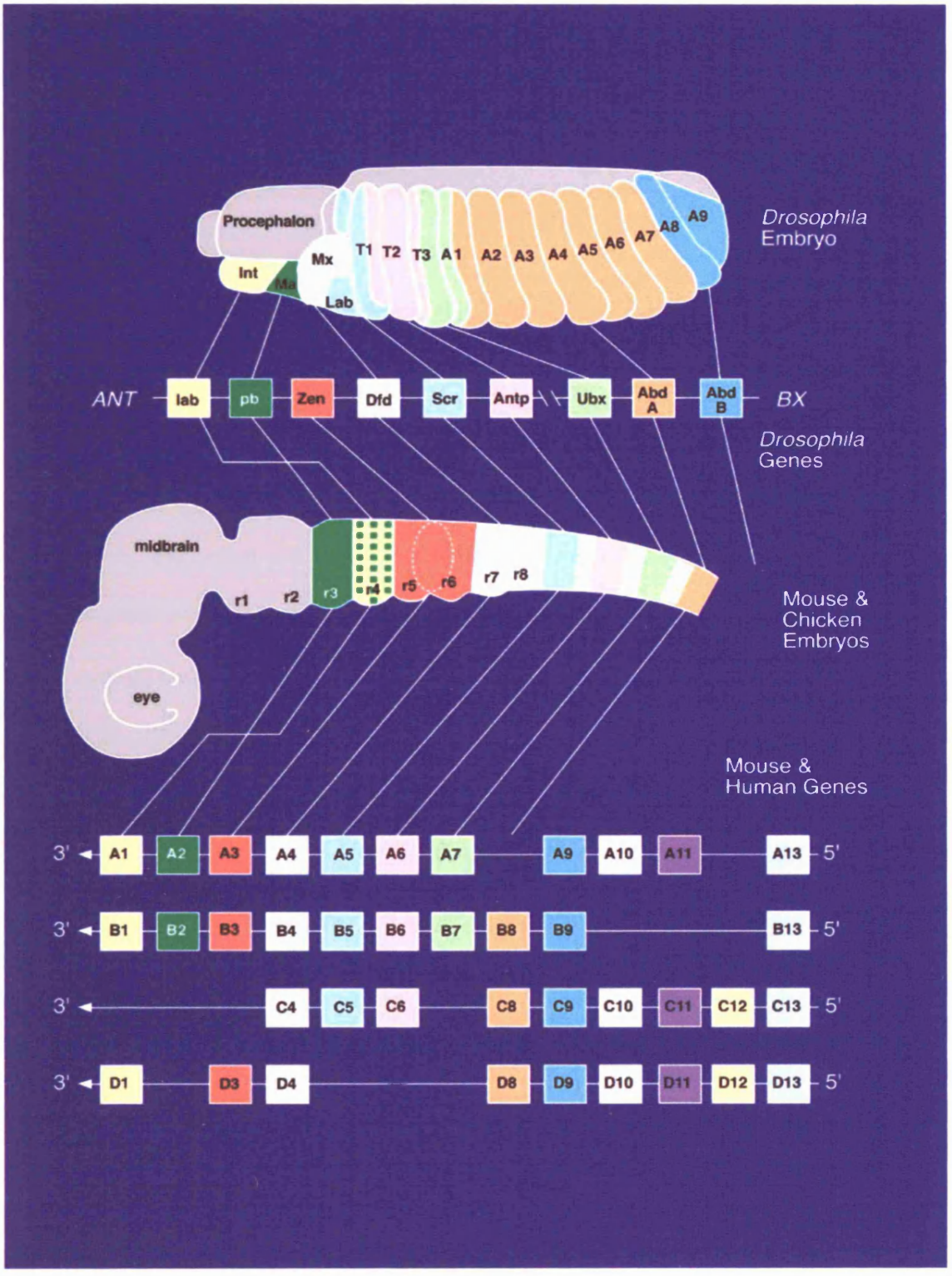


Fig. 2 : Hox gene expression domains in Drosophila and vertebrates.

Diagram representing the Drosophila and vertebrate Hox clusters and the anterior limit of their expression domains (courtesy of Alex Gould, after McGinnis and Krumlauf, 1994). Paralogous genes are shown in the same colour. Hox genes are expressed in a nested pattern, with 3' genes expressed more anteriorly than 5' genes.

has provided insights into evolutionary mechanisms, showing how gene duplications, function shuffling, and sub-functionalisation can act during evolution (Jozefowicz et al., 2003; McClintock et al., 2001; McClintock et al., 2002).

Another important aspect of hindbrain patterning is the question of how Hox gene expression is regulated. Enhancer analysis of many Hox genes has shown that different factors control the expression of each gene at different times in development (Gould et al., 1998; Manzanares et al., 2001; Marshall et al., 1994; Packer et al., 1998). In several cases, induction and early Hox gene expression is directly regulated by retinoic acid receptors, as retinoic acid response elements are present in Hox gene enhancers (Dupe et al., 1997; Gould et al., 1998; Maconochie et al., 1996; Marshall et al., 1996; Marshall et al., 1994; Packer et al., 1998). Blocking retinoic acid signalling either by removing the enzyme required for retinoic acid synthesis, or by using antagonists to retinoic acid receptors causes anteriorisation of the hindbrain (Dupe and Lumsden, 2001; Niederreither et al., 2000). This is thought to reflect collinear differential sensitivities of 5' and 3' Hox genes to increasing levels of retinoic acid (Papalopulu et al., 1991). Later acting enhancers are regulated by the segmentation genes, such as Krox20 and Kreisler, thus coupling Hox expression to segmentation. For instance, Krox20 controls *hoxa2* and *hoxb2* expression directly (Nonchev et al., 1996; Sham et al., 1993; Vesque et al., 1996), while Kreisler regulates *hoxa3* and *hoxb3* (Manzanares et al., 1999; Manzanares et al., 1997). Hoxa1 (or its functional homologue in zebrafish, Hoxb1b) is required to set the anterior limit of expression of *hoxb1* (*hoxb1a* in zebrafish) at the r3/r4 interface (Barrow et al., 2000; McClintock et al., 2002). Finally, expression is

maintained and refined by auto-regulation by Hox genes themselves, which bind, in conjunction with Meis and Pbx proteins, to enhancer elements on their own promoters (Di Rocco et al., 1997; Manzanares et al., 2001; Packer et al., 1998; Popperl et al., 1995; Popperl and Featherstone, 1992). Cross-regulation between Hox genes, such as that mentioned above between Hoxa1 and Hoxb1, acts by activating transcription at sites which are later required for autoregulation (Di Rocco et al., 1997; Popperl et al., 1995; Studer et al., 1998). Similar auto- and cross-regulation occur for the maintenance of *Hoxa3* expression in r5 and r6 (Manzanares et al., 2001).

Compartments and restrictions to cell mixing in the hindbrain.

One of the key features of *Drosophila* segmentation is that cells from adjacent parasegments do not mix with each other (Martinez-Arias and Lawrence, 1985). This ensures that parasegments remain as coherent units, and that the interfaces they form are sharp. Such units between which cells do not mix are termed compartments (Garcia-Bellido et al., 1973).

Studies in which cells were labelled before and after boundary formation in the hindbrain revealed that rhombomere boundaries restrict clonal expansion (Fraser et al., 1990). This suggests that rhombomeres constitute compartments. Another important observation is that mixing between cells of different rhombomeres displays a two-segment periodicity : cells from r2, r4 or r6 can mix with each other, as can cells from r3 and r5, but cells from even-numbered rhombomeres do not mix with cells from odd-numbered rhombomeres (Guthrie et al., 1993; Wizenmann and Lumsden, 1997).

A potential mechanism for mediating the inhibition of cell mixing between adjacent rhombomeres was suggested by the segmental expression of the genes of the Eph family of receptor tyrosine kinases (Becker et al., 1994; Gilardi-Hebenstreit et al., 1992; Nieto et al., 1992) and their transmembrane ephrinB ligands (Bergemann et al., 1995; Flenniken et al., 1996; Gale et al., 1996). These genes are expressed in complementary patterns in many species and receptor-ligand pairs can be found that define most segment interfaces in mouse, chick, zebrafish and *Xenopus* (Fig.1B, Cooke and Moens, 2002; Irving et al., 1996a; Pasini and Wilkinson, 2002; Xu et al., 2000).

The role of Eph receptors and ephrinB proteins in mediating cell sorting and affinity differences has been demonstrated in several experiments. Interfering with Eph receptor function results in *krox20*-expressing cells being found in even-numbered segments, possibly due to failure of cells to respect segment boundaries, (Xu et al., 1995). *In vitro* experiments show that bidirectional signalling between Eph receptors and ephrins leads to the segregation of Eph- and ephrin-expressing cells (Mellitzer et al., 1999). Moreover, mosaic expression of Eph receptors leads to sorting of cells to boundaries in even-numbered rhombomeres, whereas mosaic expression of ephrins results in sorting to boundaries in odd-numbered rhombomeres, indicating a role of Eph-ephrin signalling in regulating rhombomere-specific affinities (Xu et al., 1999). Indeed, some Eph receptors have been found to be under direct transcriptional control of segmentation or segment identity genes, providing an essential link between segmentation and the specification of different affinities between segments. For instance, the expression of *EphA4* and *EphA7* is regulated by Krox20 and Hoxa2, respectively (Chen and Ruley, 1998;

Taneja et al., 1996; Theil et al., 1998). Similarly, *ephb4* is downstream of *valentino* in the zebrafish and underlies the inability of *valentino* mutant cells to contribute to r5 and r6 (Cooke et al., 2001).

Thus, the segmentation of the hindbrain is maintained by the restriction of cell intermingling between rhombomeres, at least in part mediated by Eph/ephrin signals, due to complementary expression of these genes in adjacent segments. However, it must be noted that the restrictions on cell movement between rhombomeres refer only to cells in the ventricular zone. For instance, certain neurons undergo active migration between rhombomeres, in particular facial branchiomotor neurons of the VIIth nerve, from their birthplace in r4 to more posterior locations (reviewed in Chandrasekhar, 2004). Moreover, although morphologically, rhombomeres are only present transiently, restriction to cell mixing is maintained in the ventricular zone after they are no longer visible, while clonal dispersal is much greater in the mantle zone (Wingate and Lumsden, 1996). This reflects that compartmentalisation of the hindbrain stabilises patterns of gene expression in the ventricular zone which are important for neural specification, while migrations in the mantle zone can alter the final distribution of neuronal cell types.

Compartmentalisation occurs relatively late during development ; boundaries form after rhombomeres are molecularly defined (Cooke and Moens, 2002), yet clonal restriction occurs only after boundary appearance (Fraser et al., 1990). Moreover, segmental gene expression domains are initially fuzzy, and occasionally cells expressing an odd-numbered marker are found in even-numbered rhombomeres (Irving et al., 1996b). A potential explanation for how sharp interfaces and homogeneity of cell identity within segments is obtained is

provided by the observation that isolated cells, but not groups of cells, surrounded by cells of a different rhombomere can switch identity to adopt that of their neighbours (Schilling et al., 2001; Trainor and Krumlauf, 2000a). The signalling pathway that regulates this fate switching is not known, but it has been shown that Krox20 can induce its own expression non-autonomously, suggesting that it regulates a signalling molecule responsible for inducing odd-numbered identity (Giudicelli et al., 2001).

A distinct cell type at interrhombomeric interfaces : rhombomere boundaries.

Compartment boundaries were first uncovered in the wing imaginal disc of *Drosophila*, where cell lineage boundaries were found, which had no obvious morphological correlates (Garcia-Bellido et al., 1973). It was hypothesised that the role of these boundaries was to restrict the domain of action of selector genes (Garcia-Bellido, 1975). Subsequently, theoretical work proposed that boundaries could act as secondary organising centres and serve to impart positional information (Meinhardt, 1983a; Meinhardt, 1983b). It has since been demonstrated that cells at the anterior-posterior and dorso-ventral boundaries of the wing imaginal disc of *Drosophila* have specific boundary identity and secrete morphogens that pattern the wing disc over long distances (reviewed in Irvine and Rauskolb, 2001; Strigini and Cohen, 1999).

In the vertebrate hindbrain, interfaces between adjacent segments are initially fuzzy and diffuse, but sharpen over time to become sharp and straight,

both in mouse (Irving et al., 1996b) and zebrafish embryos (Cooke and Moens, 2002). Boundaries are then visible as constrictions in the neural tube, and are subsequently identifiable as a separate cell population due to specific properties, including histology, cell behaviour and gene expression (Guthrie et al., 1991; Heyman et al., 1995; Heyman et al., 1993; Layer and Alber, 1990; Mahmood et al., 1995; Mahmood et al., 1996). Thus, in this thesis, I will maintain a conceptual difference between interfaces, which are areas where gene expression domains abut, and boundaries, which are the cell population induced at interfaces, and are distinct from other cells of the adjacent segments.

Identification of boundary cells : how and when do they form ?

Morphology initially enabled the identification of rhombomere boundaries, and is still considered to be a hallmark of boundary cell specification. Rhombomeres are visible as a series of bulges in the neural tube, separated by constrictions, which constitute the boundaries (Lumsden and Keynes, 1989; Vaage, 1969). An interesting aspect of the morphological appearance of boundaries is that it allows to estimate the timing of the molecular events leading to boundary cell induction, and the relative order of formation of different rhombomeres. There are differences between species in the order of boundary formation, at least in the posterior : in chick embryos, the r5/r6 boundary is the first to form, followed by the r4/r5 and r6/r7 boundaries (Vaage, 1969), whereas in zebrafish, the r5/r6 boundary is the last to form, and the boundaries of r4 are the first (Maves et al., 2002; Moens et al., 1998). This may suggest that the mechanisms of segmentation differ between species, as there is evidence for a "proto-segment" being the precursor of r5 and r6 in zebrafish

(Cooke et al., 2001; Moens et al., 1998), which is not consistent with observations of "primary rhombomeres" in the chick hindbrain, where r4/r5, and r6/r7 appear to have a common precursor (Vaage, 1969).

Experiments in chick embryos have shown that boundaries form whenever odd- and even-numbered rhombomeres are juxtaposed (Guthrie and Lumsden, 1991). However, apposition of rhombomeres of the same parity does not form a new boundary (with the exception of r5 and r7, whose juxtaposition leads to boundary formation). This implies that an interaction between adjacent segments is responsible for boundary formation, and therefore that boundary formation is a consequence of segmentation, rather than the opposite. Moreover, the interaction involves properties that vary with segmental parity, similar to the affinity differences described above. Thus, Eph/ephrin signalling is an attractive candidate for mediating boundary formation (Cooke and Moens, 2002). Indeed, disruption of Eph/ephrin signalling leads to a loss of boundary markers in the zebrafish hindbrain (Xu et al., 1995). Nevertheless, a direct input of Eph/ephrin signalling into boundary cell induction has not been demonstrated, and the signalling events leading to boundary cell formation are still unknown.

Cellular properties of boundary cells.

Hindbrain boundary cells differ in many ways from their neighbours. They exhibit a specific, elongated morphology, and in the chick, are found in fan-shaped arrays (Heyman et al., 1993; Moens et al., 1998). Moreover, enlarged extracellular spaces are present at rhombomere boundaries, which in chick and mouse embryos are colonised by axons (Heyman et al., 1993; Lumsden and Keynes, 1989; McKay et al., 1994). It has been suggested that the enlarged

spaces between boundary cells are due to repulsive interactions at interfaces, mediated by Eph/ephrin signalling (Lumsden, 1999). Furthermore, whereas cells throughout rhombomere centres communicate through gap junctions, boundary cells are isolated from their neighbours (Martinez et al., 1992). This is consistent with the finding that Eph/ephrin signalling inhibits gap junctional communication at interfaces (Mellitzer et al., 1999). It is also possible that boundary cells have different affinity properties to cells in the centre of rhombomeres, as they do not express N-CAM, which is present throughout the ventricular zone in the centre of rhombomeres (Lumsden and Keynes, 1989). Work carried out in the Wilkinson lab in parallel with that presented in this thesis has addressed the question of boundary cell-specific affinity, and the results will be presented in the discussion. Finally, the extracellular matrix at boundaries differs from that in the centre of rhombomeres (Heyman et al., 1995; Lumsden and Keynes, 1989). Boundary matrix is enriched in laminin and chondroitin sulphate proteoglycan, suggesting a different cellular environment at boundaries than in rhombomere centres.

Boundary cells also have a different behaviour in relation to the cell cycle (Guthrie et al., 1991). First, they divide at a slower rate than cells in the centre of rhombomeres. Secondly, the nuclei of boundary cells do not undergo interkinetic nuclear migration, which is a process in which the nucleus of neuroepithelial cells moves from the ventricular surface to the pial surface of the epithelium as the cell enters S-phase, and back to the ventricular surface, where mitosis occurs. Thus, in a short pulse of BrdU labelling, which labels cells in S-phase, the nuclei of most neuroepithelial cells are located adjacent to the pial surface. The nuclei of boundary cells, however, are closer to the ventricular surface during S-phase.

These observations suggest that boundary cells may have a stabilising role at interfaces by being less motile and dividing slower than cells in rhombomere centres.

Gene expression defines boundary cells as a separate cell type.

The properties that distinguish boundary cells from cells in the centre of rhombomeres presumably reflect that gene expression differs between these two cell populations. Indeed, certain genes thus define hindbrain boundary cells as a separate cell population. I will not list all the genes whose expression has been detected at hindbrain boundaries, but I will describe certain genes, either because they have often been used as markers of boundary cells, or because they suggest potential pathways controlling boundary formation or roles of boundary cells. In particular, genes whose boundary expression is conserved between species may underlie important functions of boundaries. The homeobox gene *pax6* is upregulated at high levels in boundaries both in chick and zebrafish embryos (Heyman et al., 1995; Xu et al., 1995). Other genes encoding transcription factors expressed in boundaries include members of many gene families, such as PLZF, a zinc finger transcription factor, in both chick and mouse (Cook et al., 1995), NSCL1, a basic helix-loop-helix transcription factor, also with boundary expression conserved between chick and mouse (Theodorakis et al., 2002), and the forkhead box-containing *foxb1.2*, whose boundary expression has only been described in zebrafish embryos (also called *mariposa* and *foxb3*, Moens et al., 1996; Odenthal and Nusslein-Volhard, 1998). Another marker of interest is a signalling factor, *fgf3*, which is expressed in boundaries both of mouse and chick hindbrains, but not zebrafish (Furthauer et al., 2001; Mahmood et al., 1995;

Mahmood et al., 1996), suggesting that, in mammalian and avian embryos at least, boundaries may be signalling centres.

Patterning segments : segmentation and signalling centres.

What is a signalling centre ?

During development, complex patterns of differentiation are generated from initially equipotential fields of cells. Often, this involves setting up gradients of positional information, that instruct cells to differentiate according to their position within the field. Such gradients require reference points, and compartment boundaries are good candidates for this role (Meinhardt, 1983b). Work in *Drosophila* has suggested that there may be a recurrent theme in many different developmental sequences : first, morphogen gradients specify several cell fates, which are then determined by heritable expression of selector genes, and subsequently, interactions between the specified compartments leads to morphogen expression at the interface, forming gradients that organise pattern (Lawrence and Struhl, 1996). Morphogens are secreted substances that can induce cells to adopt different fates, depending on the concentration of morphogen they are exposed to (Wolpert, 1969).

Such mechanisms are clearly at work in organising the development of the wing of *Drosophila* (reviewed in Lawrence and Struhl, 1996). The selector genes *apterous* and *engrailed* define the dorsal and posterior compartments, respectively. Short range interactions between compartments induce the expression of *wingless* and *decapentaplegic* at the dorso-ventral and anterior-

posterior boundaries. There is now considerable evidence that these two signalling molecules act as morphogens, patterning the entire prospective wing blade along two of the three axes (for review, see Irvine and Rauskolb, 2001; Strigini and Cohen, 1999). Thus, complex patterns of vein and bristle differentiation are reliably reproduced by signalling centres, or organisers, which pattern the wing imaginal disc. It is worth noting that, in order to ensure that the correct pattern is established, it is essential that the interface at which the signalling source is induced be sharp and straight, and that the number of signalling cells be precisely regulated (Dahmann and Basler, 1999). Mechanisms for maintaining sharp interfaces and for regulating organiser cell number have been described for both the anterior-posterior and dorso-ventral boundaries. It should be noted, however, that not all signalling centres are linked to compartment boundaries, for instance, the leg imaginal disc in *Drosophila* is patterned by opposing gradients of Wingless and Decapentaplegic, but no restrictions to cell mixing are observed, and the appearance of sharp expression domains is due to cross-repression of the two pathways along the dorso-ventral axis, and their cooperation along the proximo-distal axis (Irvine and Vogt, 1997; Lecuit and Cohen, 1997).

Signalling at parasegment boundaries in Drosophila.

A further example of how signalling centres organise tissue is the precise arrangement of hairs, or denticles, in the cuticle of the *Drosophila* larva (reviewed by Sanson, 2001). Each segment consists of denticle belts and smooth cuticle, both about six cells in width. Denticle belts are made up of six rows of individually identifiable denticles. This cuticle pattern reflects the fact that the

underlying epidermis, which secretes the cuticle, is patterned, and that every epidermal cell within one segment has a distinct identity. This pattern is established during the segmentation process. A signalling centre is established at parasegment boundaries, with the anterior row of cells expressing *wingless*, and the two cells posterior to the interface expressing *hedgehog* and *engrailed*. The range of Wingless signalling is asymmetric, acting on cells four diameters away in the anterior direction, but only one cell posteriorly (Sanson et al., 1999). Thus, six cells, including the Wingless-secreting cell, are exposed to Wingless. Transduction of the Wingless signal leads to the repression of *shavenbaby*, which is a selector gene required for denticle secretion, and therefore the cells exposed to Wingless secrete smooth cuticle (Payre et al., 1999). Cooperation of Wingless and Hedgehog signalling also serves to position a stripe of three cells expressing *rhomboid*, whose product is required for the activation of an Epidermal Growth Factor-related ligand, Spitz, whose activity regulates denticle formation. Finally, Wingless, Spitz and Hedgehog each play a role in activating three discrete cell rows of expression of *stripe*. These cells will give rise to the tendons attaching the muscles to the epidermis (Sanson, 2001).

This brief description shows how segmentation, by leading to the establishment of signalling centres at parasegment boundaries, leads to fine patterning of cells. In more general terms, this is an example of how subdivisions of the embryo give rise to a complex and refined pattern of differentiated cell types, through the formation of organisers.

Signalling centres pattern the vertebrate neural tube.

In vertebrates, subdivision and patterning of tissue also involves signalling centres. For instance, anterior-posterior patterning of the limbs, and consequently digit determination, involves a signalling centre known as the Zone of Polarising Activity (ZPA), located at the posterior side of the growing limb bud. A homologue of the *Drosophila hedgehog*, *Sonic Hedgehog*, is expressed in the ZPA and behaves as a morphogen, inducing different digits at different concentrations (reviewed in Capdevila and Izpisua Belmonte, 2001). Another signalling centre in the limb, the Apical Ectodermal Ridge (AER), corresponds to borders of lineage restriction (Kimmel et al., 2000), and secretes Fibroblast Growth Factors (FGFs) that pattern limb development along the proximo-distal axis (Capdevila and Izpisua Belmonte, 2001).

Several organisers pattern the developing vertebrate neural tube (see Fig.4A). The roof plate and floor plate run along the length of the neural tube, dorsally and ventrally, respectively. They secrete morphogens, such as the ventralising Sonic Hedgehog, or dorsalising Bone Morphogenetic Proteins (BMPs, homologues of *decapentaplegic*, and members of the large Transforming Growth Factor β family), and pattern cell types along the dorso-ventral axis (see below). Regionalisation of the neural tube along the anterior-posterior axis also involves signalling centres (reviewed in Echevarria et al., 2003). As mentioned previously, rhombomere 4 is a source of FGFs and specifies and patterns the adjacent rhombomeres in zebrafish (Maves et al., 2002; Walshe et al., 2002), and is required for expression of specific genes in r3 and r5 in chick (Graham and Lumsden, 1996). Recent work has uncovered a new signalling centre at the boundary between thalamus (or dorsal thalamus) and prethalamus (or ventral

thalamus) : the *zona limitans intrathalamica* (ZLI, Kiecker and Lumsden, 2004). The ZLI itself is a compartment ; cells of the ZLI do not cross either into the thalamus or the prethalamus (Larsen et al., 2001; Zeltser et al., 2001). The ZLI expresses the morphogen *Sonic Hedgehog* (*shh*), which regulates gene expression in both thalamus and prethalamus. For instance, *nkx2.2* is expressed in a domain immediately flanking the ZLI on either side, while *dlx2* is expressed in the whole prethalamus and *gbx2* only in the thalamus, but all three genes require Shh for their expression. Differential responses to Shh are mediated by *irx3*, which seems to act as a selector gene for thalamic identity (Kiecker and Lumsden, 2004).

One of the most prominent and well studied organisers in vertebrates is the isthmic organiser, which forms at the midbrain-hindbrain boundary (MHB, reviewed in Echevarria et al., 2003; Wurst and Bally-Cuif, 2001). Positioning of the MHB depends on interactions between the midbrain, expressing genes of the Otx family, and the anterior hindbrain, which expresses Gbx family members, but other factors appear to be involved in the induction of the MHB. In the chick, the isthmus does not appear to be a boundary of lineage restriction, and it is likely that the maintenance of sharp gene expression interfaces is due to cells switching fate according to their position relative to the MHB (Jungbluth et al., 2001; reviewed by Pasini and Wilkinson, 2002). Ablation and grafting studies have shown that the isthmic organiser is necessary and sufficient for the development of the whole of the midbrain, and of the anterior hindbrain (particularly of rhombomere 1 and its derived structures, such as the cerebellum). The isthmic organiser expresses *wnt1* (founding member of the Wnt family, homologous to *Drosophila wingless*)and *fgf8*. Of these signals, only FGF8 is able

to mimic the patterning properties of the organiser. Finally, as well as being responsible for maintaining the midbrain-hindbrain area, signals from the isthmic organiser also pattern this region. For instance, FGF8 from the organiser cooperates with signals from the floor plate (and also presumably with selector genes specifying midbrain or r1 identity) to induce midbrain-specific dopaminergic neurons and r1-specific serotonergic neurons of the raphe nuclei, and noradrenergic neurons of the *locus coeruleus* require dorsal BMP signals as well as isthmic-derived FGF (Wurst and Bally-Cuif, 2001).

In summary, organisers along the length of the neural tube pattern the dorso-ventral axis, while several signalling centres are involved in patterning specific regions of the anterior-posterior axis, providing the head region of vertebrate embryos with a grid-like coordinate system.

The control of neurogenesis.

The role of patterning of neural tissue is to ensure that the correct number and subtypes of neurons and glia are born in appropriate locations, and that these neurons accurately connect to each other, so as to generate functional circuits. Thus, the subdivision of the neural epithelium must be linked to the control of neurogenesis.

Therefore, in order to understand the patterning of a neural tissue, it is important to understand how it relates to the spatial and temporal generation of neurons and glia and of different neuronal types. There are two fundamental, but interlinked questions to address : first, how neurons are produced, and second, how different types of neurons are produced.

A general molecular pathway for neurogenesis.

A conserved gene family controls many aspects of the acquisition of a generic neuronal fate. Members of the basic helix-loop-helix (bHLH) family of transcription factors encoded by proneural genes act to induce neuronal differentiation, controlling downstream bHLH neuronal differentiation genes, while other bHLH proteins counteract proneural proteins and maintain cells in an undifferentiated state (Bertrand et al., 2002). These opposing factors are linked by a process called lateral inhibition, involving signalling through the Notch pathway.

Making neurons : the* achaete-scute *and* atonal *gene families.

Proneural genes are involved in driving cells towards neuronal differentiation (reviewed in Bertrand et al., 2002). These genes were first identified in *Drosophila* as being required for the formation of subsets of sensory bristles and sense organs. Genes of the *achaete-scute* complex and others related to *atonal* are each required for the generation of different neural progenitors from ectodermal cells, while gain of function experiments show that they can induce ectopic neural cells when expressed in the ectoderm, establishing a paradigm for these genes acting as selector genes for neural development.

Vertebrate homologues of proneural genes have been identified, and are divided into several families. The *ash* gene family is homologous to *achaete-scute* and the *ath* and *neurogenin* (*ngn*) families are related to *atonal*. To date, only three genes have been found to have proneural activity in mammals : *Mash1*, *Ngn1* and *Ngn2* (Fode et al., 1998; Guillemot et al., 1993; Ma et al., 1998). Loss of function analyses show that proneural genes are required for neurogenesis, and that in their absence, neuronal populations are lost and Notch signalling (see below) is not initiated. There are differences between proneural function in *Drosophila* and vertebrates, however, as in vertebrates, these genes function in the context of a neural tissue to specify progenitors that are limited to a neuronal fate (Bertrand et al., 2002). Moreover, loss of proneural genes leads to an increase in gliogenesis, implicating proneural genes in the decision between neuronal versus glial fate (Nieto et al., 2001). In zebrafish, only one *neurogenin* gene, *ngn1* has been identified, but two *Mash1* homologues, *asha* and *ashb*, are

present (Allende and Weinberg, 1994; Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998).

The action of proneural genes has been extensively studied in the mammalian cortex, as well as in *Xenopus* and zebrafish embryos. They act in many ways : first, they initiate lateral inhibition to single out neuronal progenitors from neuroepithelial stem cells. Second, positive feedback loops, by direct regulation of their own promoters, or indirectly, via the regulation of factors such as *hes6* or *coe2*, increase levels of proneural gene expression, leading to irreversible commitment of the cell. Finally, they induce expression of genes of the *neuroD* family of neuronal differentiation genes, which are also bHLH genes related to *atonal* (Bertrand et al., 2002; Ross et al., 2003). The differentiation process is linked to exit from the cell cycle, but it is not known whether proneural genes themselves regulate cyclin-dependent kinase inhibitors, or if downstream differentiation genes are responsible.

The regulation of proneural gene expression and activity is achieved in many ways. Little is known about how proneural gene expression is positively induced in the vertebrate nervous system, but in *Drosophila*, promoter elements have been described for different expression domains, showing that several signalling inputs are integrated to establish proneural gene transcription (Gomez-Skarmeta et al., 2003). The activity of proneural genes is also tightly regulated. For instance, Mash1 activity can be regulated by phosphorylation by glycogen synthase kinase 3 (GSK3), an enzyme that is regulated by several intercellular signalling pathways (Moore et al., 2002). Another mechanism of proneural gene activity regulation is the availability of binding cofactors. Proneural proteins bind DNA as heterodimers with other bHLH family members, E proteins, which are

ubiquitously expressed. However, Id genes, which have a helix-loop-helix domain, but lack the basic DNA binding domain, can act in a dominant negative manner, by sequestering E proteins (reviewed in Ross et al., 2003). Id proteins therefore counteract proneural gene activity, and knockout of these genes leads to premature neuronal differentiation (Lyden et al., 1999). Finally, both expression and activity of proneural genes is inhibited by another class of bHLH genes, the *hairy/enhancer of split* family (Ross et al., 2003). These genes are the effectors of Notch signalling during lateral inhibition.

Keeping cells undifferentiated : the Notch pathway and lateral inhibition.

Lateral inhibition is a process whereby a cell selected to adopt a particular fate inhibits its neighbours from doing the same (Fig3). It was first described in *Drosophila*, where loss or gain of function mutations lead to supernumerary or loss of neural precursors (Heitzler and Simpson, 1991). Lateral inhibition involves signalling through the Notch receptor and its ligands Delta and Serrate/Jagged. Both receptor and ligand are transmembrane proteins. Upon interaction, the ligand is internalised by the signalling cell, provoking cleavage of the intracellular domain of Notch, which translocates to the nucleus and binds to its partner, Suppressor of Hairless (also called CSL) to regulate gene expression (reviewed in Schweisguth, 2004).

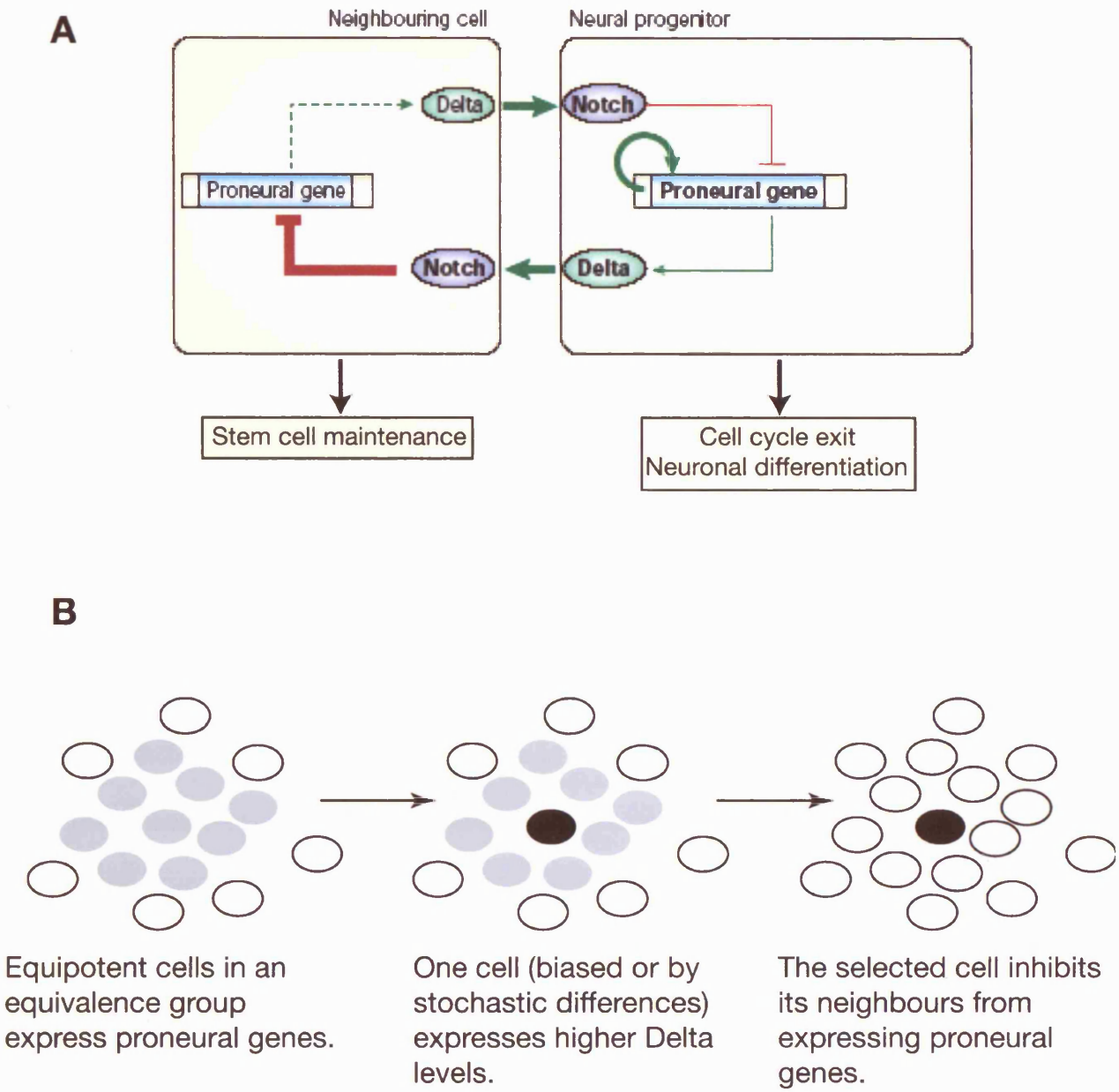


Fig. 3 : Neurogenesis and lateral inhibition.

A : Molecular pathway of lateral inhibition (adapted from Bertrand et al., 2002). Proneural genes positively regulate their own expression and that of the Notch ligand delta (green arrows). Delta signals to Notch in adjacent cells, which represses proneural gene expression in the receiving (left) cell. This effect is reinforced over time as the signalling cell receives less inhibition, as Delta expression is downregulated in the receiving cells. The signalling cell initiates a programme of neuronal differentiation, while its neighbours remain undifferentiated. B : Selection of a neural precursor from an epithelial field of cells. Groups of cells (called equivalence groups) express equal levels of proneural genes. Lateral inhibition ensures that only one cell is selected to become a neural precursor.

For lateral inhibition to occur, it is necessary that the outcome of Notch signalling is to repress expression of *delta* and *serrate* (Lewis, 1998). In the context of neurogenesis, expression of the Notch ligands is driven by proneural genes (Fig.3A, Bertrand et al., 2002). Low level expression of proneural gene in a group of equipotential cells leads to low levels of Notch ligand expression, and activation of the Notch receptor. If one cell in the group expresses higher levels of ligand, either randomly or by some bias, it will activate the Notch pathway more strongly in the neighbouring cells, causing them to express less ligand, and thus decreasing Notch activation and increasing ligand expression in the former cell (Fig.3B). Combined with positive feedback of proneural genes on their own expression, this leads to the amplification and maintenance of stochastic or biased differences, and to the selection of a single cell from a group that expresses high levels of *delta* and proneural genes, which is thus fated to become a neural progenitor (Artavanis-Tsakonas et al., 1999; Bray, 1998; Lewis, 1998; Simpson, 1997).

Activation of the Notch pathway in the adjacent cells leads to the expression of *hairy/enhancer of split* genes (*hes, hey* and *her* gene families in vertebrates), which repress proneural genes and inhibit neuronal fate (Ross et al., 2003). For instance, recent work has shown that in a *hes1/hes5* mouse mutants, neuroepithelial stem cells are not maintained and differentiate precociously and ectopically into neurons (Hatakeyama et al., 2004). This is similar to the zebrafish mutant, *mind bomb*, where Notch signalling is severly impaired, and where early-born neurons are generated in vast excess, while later types of neurons are absent, due to a loss of progenitors (Bingham et al., 2003; Itoh et al., 2003; Park and Appel, 2003). Thus, Notch activation is crucial in the vertebrate

neural tube to maintain stem cells and prevent differentiation from occurring at the wrong time or place. Although its role in neurogenesis is the best characterised, the Notch pathway has many other roles during development, including boundary formation, lineage decisions, cell proliferation and segmentation of the mesoderm in vertebrates (reviewed in Artavanis-Tsakonas et al., 1999; Bray, 1998; Lewis, 1998).

Generation of neuronal diversity and patterning of neuronal types.

The molecular pathways described above give insights into the general acquisition of neuronal properties, but do not explain how different types of neurons are obtained. Neurons are the most diverse cell population in vertebrates. For instance, in the spinal cord, there are motor neurons, sensory neurons and several types of interneurons (reviewed in Jessell, 2000). Considering only motor neurons, different groups can be distinguished, depending on the area they innervate, which are grouped into columns, for instance, the medial motor column neurons that innervate limbs. Within each column, motor neurons are separated into medial and lateral divisions, and within each division, motor neurons are found in pools that correspond to the neurons that innervate single muscle groups. Each type of neuron thus defined by morphology and axon projection has a distinct molecular identity.

Several mechanisms are responsible for such diversity among neurons. One such mechanism is the regulation of the temporal identity of progenitors,

such that they generate different types of neurons over time. This can be achieved as a result either of intrinsic timing mechanisms or of changing extrinsic cues (Pearson and Doe, 2004). Examples of both types of temporal regulation exist : neuroblasts in *Drosophila* give rise to different neuronal precursors at every division, due to an intrinsic programme of changing transcription factor expression (Pearson and Doe, 2003). In the mammalian cortex, however, heterochronic transplants show that cells can adopt a later fate if progenitors are transplanted before they start a division cycle, indicating the presence of extrinsic cues regulating neuronal identity (McConnell and Kaznowski, 1991). Conversely, cells from older donors are restricted in the cell types they can produce, even in young hosts, implying that an intrinsic mechanism also occurs (Desai and McConnell, 2000; Frantz and McConnell, 1996). A combination of both extrinsic and intrinsic factors is also observed in the retina, where a suggested model for temporal determination of neuronal cell types is that stem cells undergo changes in intrinsic competence states to produce specific types of cell, but that extrinsic signals regulate whether they produce those cells (Livesey and Cepko, 2001). Other examples of temporal specification of neuronal cell type are found in the spinal cord, where oligodendrocytes are generated from progenitors that previously gave rise to motor neurons (Richardson et al., 2000; Rowitch et al., 2002), and in the hindbrain, where motor neuron progenitors later give rise to serotonergic neurons, except in rhombomere 4 (Pattyn et al., 2003a).

These last two examples highlight the fact that temporal determination cannot account for all neuronal diversity in the central nervous system, as temporal changes occur in subsets of progenitors that are already defined along

the dorso-ventral axis, and in the case of the hindbrain, also along the anterior-posterior axis. Thus, spatial patterning is crucial for the generation of distinct neuronal types.

Dorso-ventral patterning

One of the best understood models of neural patterning is along the dorso-ventral axis of the spinal cord (reviewed in Briscoe and Ericson, 2001; Jessell, 2000). In the spinal cord, motor neurons are found ventrally, and neurons that process sensory inputs dorsally, and several types of interneurons are found at various dorso-ventral positions. Two signalling centres, the floor plate and the roof plate, are responsible for patterning along the dorso-ventral axis. The floor plate is a specialised glial cell population at the ventral midline of the spinal cord, induced by the notochord, a mesodermal structure below the neural tube. Both the notochord and the floor plate secrete the morphogen Sonic Hedgehog (Shh). Shh induces distinct neuronal progenitor types at different concentrations. The action of Shh is mediated by the expression of two classes of homeobox proteins, the first (including members of the Pax, Dbx and Irx families) being repressed by different thresholds of Shh activity, and the second class being activated by Shh signalling, and comprising genes of the Nkx family. Class I and class II genes cross-repress each other in pairs, leading to the formation of sharp interfaces of expression. Thus, a homeodomain protein code is established, and determines five distinct progenitor domains (Briscoe et al., 2000). Cells in each domain generate a distinct class of post-mitotic neurons. However, although Shh can generate all five classes of ventral progenitors, it is not required in all of

them, and instead, retinoids derived from paraxial mesoderm induce the two most dorsal types of ventral interneuron *in vivo* (Pierani et al., 1999).

Patterning of the dorsal half of the spinal cord is less well understood, in part due to fewer markers being available to define individual progenitor domains. Nevertheless, six types of dorsal interneurons can be distinguished at different dorso-ventral positions. The code for these progenitor domains appears to involve bHLH as well as homeodomain containing proteins (Bermingham et al., 2001; Chizhikov and Millen, 2005; Gowan et al., 2001; Mansouri and Gruss, 1998). Indeed, recent work indicates that, as well as their general proneural function of promoting neurogenesis, proneural genes may have direct inputs into neuronal subtype specification (Bertrand et al., 2002). Signals from the roof plate pattern the dorsal spinal cord, and include many members of the Transforming Growth Factor β family, such as BMPs and Growth/Differentiation Factors (GDFs), as well as at least two members of the Wnt family, Wnt1 and Wnt3a (reviewed in Chizhikov and Millen, 2005). Genetic ablation of roof plate cells suggests that only the three most dorsal populations of neurons are specified by roof plate signals, and one transcription factor, *lbx1*, is responsible for specifying the three remaining types (Lee et al., 2000; Muller et al., 2002). However, BMPs regulate the range of Shh signalling and prevent ventralisation of the dorsal half of the spinal cord, implying that that the roof plate does pattern more than just the three dorsal-most neuronal progenitor populations (Briscoe and Ericson, 2001; Jessell, 2000).

Thus, many (up to eleven) progenitor domains are defined along the dorso-ventral axis of the spinal cord, by the cooperation of dorsal and ventral signalling centres, as well as signals from paraxial mesoderm. These domains

each give rise to distinct classes of neurons, due to the combinations of transcription factors expressed by the progenitors. However, differences in the transcription factor code, in the gene regulatory hierarchies and in the types of neurons produced are observed at different anterior-posterior locations in the neural tube. For instance, in the spinal cord, most motor neurons are somatic motor neurons, innervating somite-derived skeletal muscles, except at particular sacral and thoracic levels, where visceral motor neurons are also generated, which innervate autonomic ganglia (Jessell, 2000). In the hindbrain, both types of motor neuron are produced, but they originate from different progenitor domains, and analysis of their regulation shows that the same homeodomain and bHLH containing proteins may be in different regulatory hierarchies compared to the spinal cord (Ericson et al., 1997; Pattyn et al., 2003a; Pattyn et al., 2003b; Takahashi and Osumi, 2002; Vallstedt et al., 2005). Thus, anterior-posterior patterning is also an important factor in the production of neuronal diversity.

Anterior-posterior patterning

Less is known about how anterior-posterior patterning affects neuronal specification than for dorso-ventral patterning. However, while Shh signalling occurs ventrally at all axial levels, responses to this signal differ according to position along the anterior-posterior axis, implying that the way in which neural cells respond to the same signal is modified by their axial properties. The dorso-ventral progenitor code differs between spinal cord and hindbrain, as mentioned above, and is also different in the midbrain and forebrain (Puelles et al., 2004; Toresson et al., 2000). Direct input from anterior-posterior signalling centres affects neuronal specification in these areas. For instance, the generation of

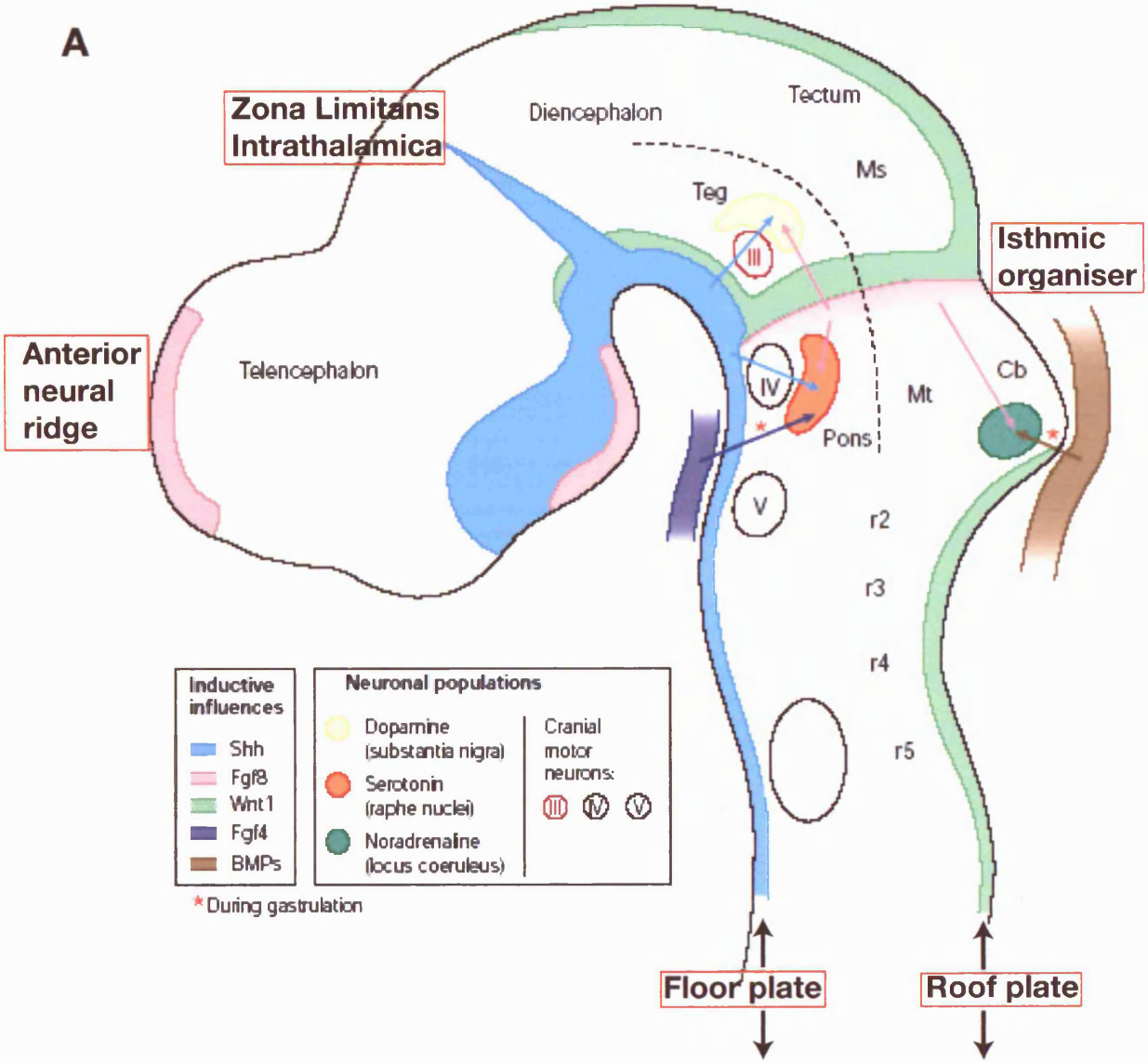
midbrain dopaminergic and r1 serotonergic nuclei requires a direct input of isthmic-derived FGF signals, as well as Shh from the floor plate (Fig.4A, Hynes and Rosenthal, 1999; Ye et al., 1998). Recent work has shown that, although the same signals are present in the anterior hindbrain and posterior midbrain, they are interpreted in different ways by cells in these tissues, due to the expression of *otx2* in the midbrain (Puelles et al., 2004). In the hindbrain, different segments give rise to different neurons (Lumsden and Keynes, 1989). One well studied example integration of anterior-posterior and dorso-ventral signalling is the case of ventral hindbrain progenitors that sequentially generate visceral motor neurons and serotonergic neurons. In rhombomere 4, this switch does not take place, due to the maintained expression of *phox2b*, which is a direct target of the r4-specific genes, Hoxb1 and Hoxb2, but *phox2b* expression also requires the dorso-ventrally restricted Nkx2.2 (Pattyn et al., 2003a; Samad et al., 2004).

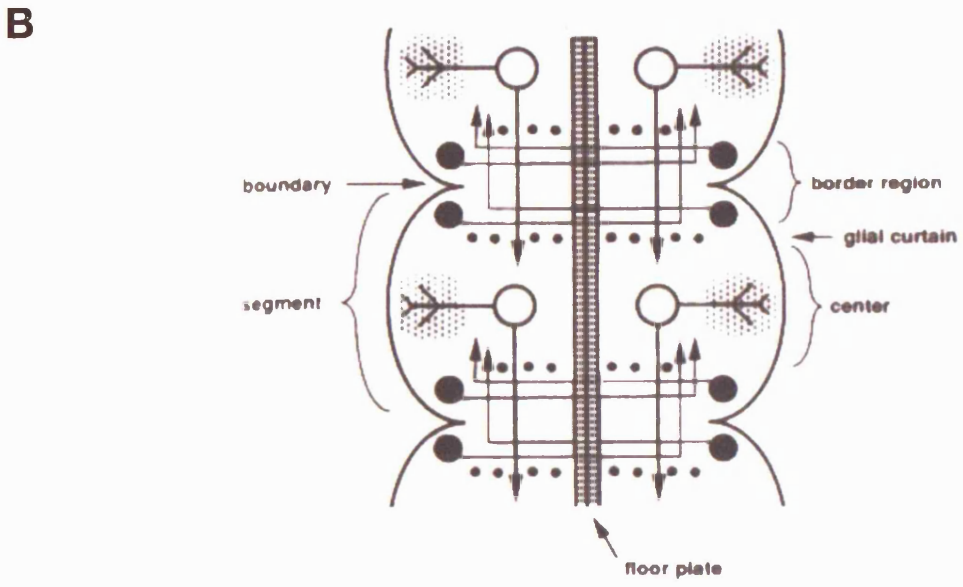
Similarly in the spinal cord, there are differences in neuronal specification along the anterior posterior axis. One of the most evident is that different motor columns form at different locations : at limb levels, lateral motor columns are generated, while in intervening thoracic regions, autonomic motor neurons are grouped into the Column of Terni in chick (Dasen et al., 2003; Jessell, 2000). The establishment of these columns is due to expression of different Hox genes at different anterior-posterior locations, which in turn is due to a posterior-to-anterior gradient of FGF signalling. Expression of either group 9 Hox genes or of group 6 Hox genes leads to those neurons adopting forelimb lateral motor column or Column of Terni identity, respectively (Dasen et al., 2003). Remarkably, this patterning event occurs in post-mitotic cells, once they have been specified as motor neurons.

Another example of anterior-posterior patterning has been described in the hindbrain of the zebrafish (Fig.4B, Hanneman et al., 1988; Trevarrow et al., 1990). The distribution of neuronal and glial cell types is patterned along the anterior-posterior axis within each hindbrain segment. Early-born neurons and reticulospinal neurons are located in segment centres, while commissural neurons are located at rhombomere boundaries. Separating centre and boundary regions, a row of radial glial cells forms a "glial curtain". In chick and mouse, individual types of neuron are generally found in longitudinal columns along the anterior-posterior axis within each rhombomere (Auclair et al., 1999; Clarke and Lumsden, 1993; Lumsden et al., 1994). Thus, the anterior-posterior patterning of neural types within each rhombomere in the zebrafish is remarkable compared to other vertebrate systems, where patterning along the anterior-posterior axis occurs between, but not within segments, making the zebrafish hindbrain a particularly interesting model for studying regional neural patterning.

Fig. 4 : Signalling centres and patterning in the central nervous system.

A : Diagram schematising the anterior vertebrate neural tube, with signalling centres indicated in red boxes (adapted from Wurst and Bally-Cuif, 2001). Secreted signalling molecules are indicated by colours, as shown in the key. Particular neuronal populations around the isthmic organiser, as described in the text, are indicated, together with arrows showing the inductive signalling events that pattern them. B : Cell type organisation along the anterior-posterior axis of each rhombomere in the zebrafish (from Trevarrow *et al.*, 1990). Reticulospinal neurons (grey) are located in the centre of rhombomeres, and commissural neurons (black) are at segment borders, separated from the centre regions by a curtain of glial fibres.


A
Zona Limitans Intrathalamica
Diencephalon
Tectum
Ms
Teg
III
Isthmic organiser
Anterior neural ridge
Telencephalon
IV
Mt
Cb
Pons
V
r2
r3
r4
r5
Inductive influences
Shh
Fgf8
Wnt1
Fgf4
BMPs
Neuronal populations
Dopamine (substantia nigra)
Serotonin (raphe nuclei)
Noradrenaline (locus coeruleus)
Cranial motor neurons:
III IV V
*During gastrulation
Floor plate
Roof plate


B
boundary
segment
border region
glial curtain
center
floor plate

The Wnt signalling pathway and its roles in development.

The Wnt pathway is named after its upstream ligands, the Wnt family of secreted signalling molecules. This name derives from the two founding members of the family (Nusse et al., 1991), the *Drosophila wingless* gene, which was identified for its role in wing development (Sharma and Chopra, 1976), and its mouse homologue, *int-1* (for integration site 1), which was identified as a potential oncogene due to the frequent insertion of a tumour-provoking virus, the Mouse Mammary Tumour Virus, leading to overexpression of its transcript (Nusse and Varmus, 1982; van Ooyen and Nusse, 1984). These two very different contexts leading to the identification of Wnt gene homologues suggest that the Wnt signalling pathway has normal roles in regulating important developmental events, and promotes carcinogenesis when deregulated.

Molecular characterisation of the Wnt pathway.

Extensive biochemical and genetic evidence (see for instance Noordermeer et al., 1994 for genetic elucidation of the pathway in *Drosophila*) has allowed the molecular pathway leading to gene transcription downstream of Wnt ligands to be characterised (Cadigan and Nusse, 1997; Logan and Nusse, 2004). Moreover, this evidence has shown that at least two distinct pathways exist downstream of Wnt ligands or receptors, although only one of these, the

"canonical" Wnt pathway (Fig.5) is well understood (Huelsken and Behrens, 2002; Veeman et al., 2003).

"Canonical" Wnt signalling.

The canonical Wnt signalling pathway activates nuclear transcription through the Tcf/Lef family (T-Cell Factor/Lymphoid Enhancer Factor – called *dTCF* or *pangolin* in *Drosophila* and *pop-1* in *C. Elegans*). Tcf/Lef family members are HMG box-containing transcription factors, and are constitutively bound to their target DNA sequences (reviewed in Roose and Clevers, 1999). In the absence of a Wnt signal, Tcfs actively repress transcription of target genes, due to interactions with the transcriptional repressor Groucho (Cavallo et al., 1998). The Wnt pathway switches Tcfs to an activator function by promoting the nuclear entry of β-Catenin (Armadillo in *Drosophila*, WRM-1 in *C. Elegans*), which forms a complex with Tcfs and provides a transcriptional activator domain (Cadigan and Nusse, 1997).

The way in which the regulation of β-Catenin localisation is achieved involves regulated degradation. There are two pools of β-Catenin in the cell, one of which is localised to the cell membrane, and is involved in cell adhesion (Nelson and Nusse, 2004), and one in the cytoplasm, which is rapidly degraded by ubiquitination-dependent proteasome degradation (Aberle et al., 1997). Wnt signalling inhibits the degradation of the cytoplasmic pool of β-Catenin, enabling it to enter the nucleus (Giarre et al., 1998). The degradation of β-Catenin is mediated by a complex involving many proteins ; of particular importance are Axin, Adenomatous Polyposis Coli (APC) and Glycogen Synthase Kinase 3β

(GSK3β, or Zeste White 3 in *Drosophila*). GSK3β phosphorylates β-Catenin to induce its ubiquitination and subsequent degradation.

Wnts signal to inhibit the degradation of β-Catenin. They bind their receptors, Frizzled proteins, and require co-receptors, LRPs (low density lipoprotein receptor-related proteins, called Arrow in *Drosophila*). This interaction activates Dishevelled, which binds to and recruits Axin to the membrane, leading to disassembly of the β-Catenin degradation complex, allowing β-Catenin to activate target genes (reviewed in Huelsken and Behrens, 2002; Logan and Nusse, 2004; Tolwinski and Wieschaus, 2004). In addition, Dishevelled acts at other levels to inhibit β-Catenin degradation.

This picture is further complicated by the many other components of the pathway that have been identified. For instance, secretion of active Wnt molecules requires a lipid modification, palmitoylation, mediated by the product of the *porcupine* gene in *Drosophila* (Kadowaki et al., 1996; Zhai et al., 2004). Heparin sulphate proteoglycans are required for either transport or reception of the ligand, and the signal can also be modulated by extracellular inhibitors, such as SFRPs (secreted frizzled-related proteins) or WIF (Wnt inhibitory factor), that bind Wnts extracellularly, or Dickkopf, that binds to the Wnt co-receptors, LRP/Arrow, as well as causing them to be removed from the cell surface. Many more components of the β-Catenin degradation pathway have been identified (for a comprehensive review of known pathway components, see Logan and Nusse, 2004 or http://www.stanford.edu/~rnusse/wntwindow.html). Moreover, both β-Catenin and Tcfs have many binding partners in the nucleus that could modulate their activity and/or the expression of specific target genes (Sharpe et al., 2001).

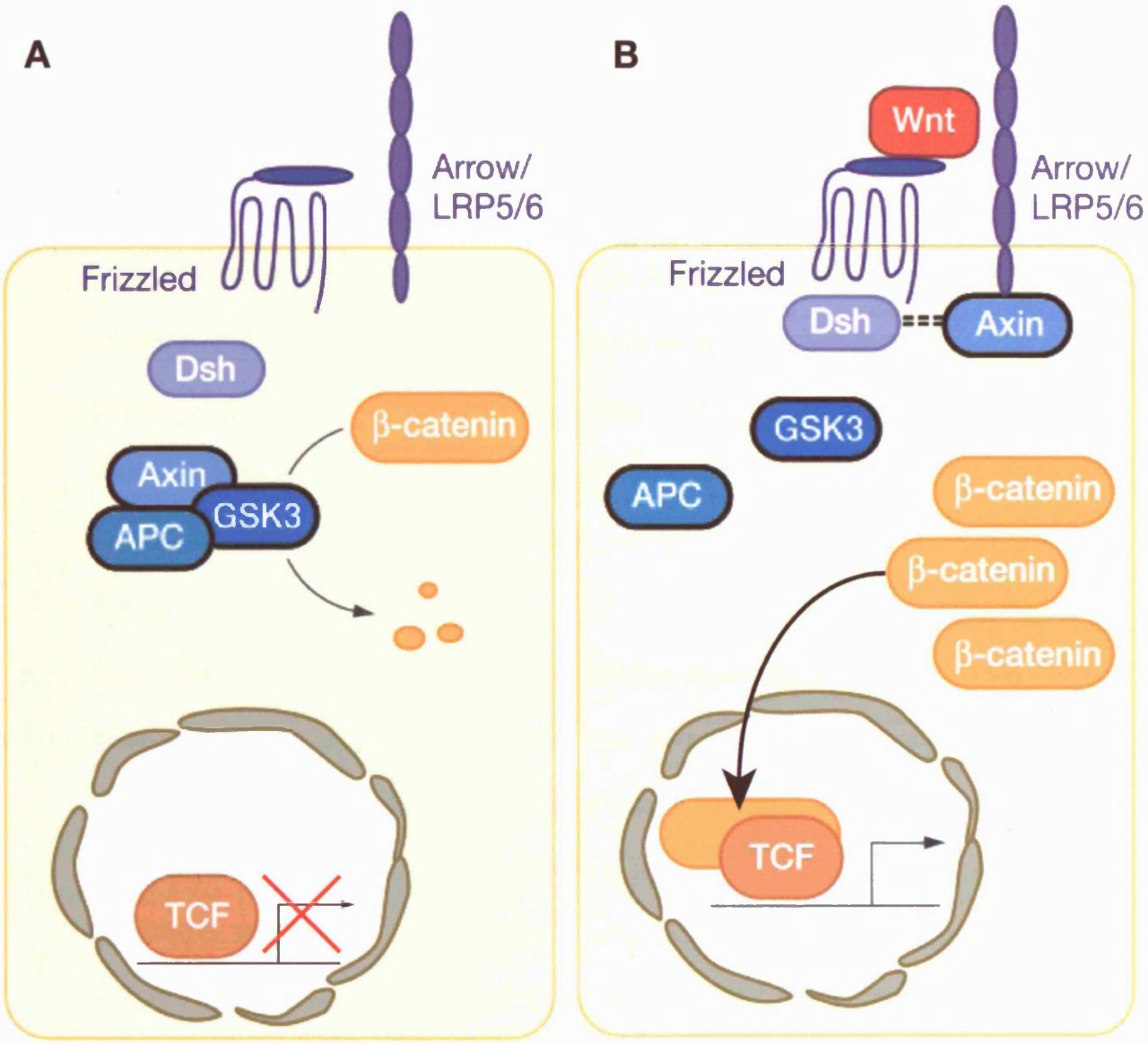


Fig. 5 : The canonical Wnt signalling pathway.

In the absence of Wnts (A), cytoplasmic b-catenin is phosphorylated by a complex including APC, Axin and GSK3, which targets b-catenin for degradation. Tcf transcription factors are bound to DNA, but repress transcription of their target genes. B : Wnt ligands bind Frizzled and interact with Arrow/LRP5/6. This activates Dishevelled, which recruits Axin to the membrane, and dislocates the b-catenin degradation complex, allowing cytoplasmic b-catenin to accumulate. b-catenin translocates to the nucleus, where it binds to Tcfs, and the b-catenin/Tcf complex activates transcription of target genes.

In summary, the canonical Wnt pathway is complex, but in its essence involves the inhibition of β-Catenin degradation upon binding of Wnts to their receptors. β-Catenin then translocates to the nucleus and associates with Tcfs to activate transcription of genes that Tcfs repress in the absence of Wnts (Fig.5).

Other signalling pathways downstream of Wnt ligands.

While canonical Wnt signalling is mediated through the regulation of cytoplasmic β-Catenin, some roles of Wnts have been found to be independent of β-Catenin (reviewed in Veeman et al., 2003). While many details of these pathways remain unclear, at least two different cellular events are affected by β-Catenin-independent signalling : polarity of cells and intracellular calcium release.

Two similar, but not identical, pathways regulating cell polarity have been described which regulate *Drosophila* planar cell polarity (PCP) and convergent extension movements during vertebrate gastrulation (Mlodzik, 2002; Veeman et al., 2003). They differ especially in that no Wnt ligand is known to be involved in PCP, whereas at least two, Wnt5a and Wnt11, regulate convergent extension movements. Frizzled and Dishevelled are involved in this pathway, and modulate the activity of certain members of the Rho GTPase family of small G-proteins. An adaptor protein, Daam1 is required for Rho activation downstream of Dishevelled, and one downstream effector of Rho, ROK, is involved both in PCP and in convergent extension. Activation of Rho and ROK leads to cytoskeletal rearrangements. Also involved are two transmembrane proteins, Strabismus (mutated in the *trilobite* zebrafish mutant, also called Vangl2 in mouse) and Flamingo (Celsr in vertebrates), and the intracellular LIM-

domain protein Prickle. These proteins are known to regulate the asymmetric location of each other and of Frizzled in the cell and establish PCP in *Drosophila* (Fanto and McNeill, 2004). They are also involved in convergent extension, but it is not known whether they are asymmetrically distributed. No clear linear pathway has been established between these proteins and the Frizzled/Dishevelled/Rho hierarchy.

The Wnt/calcium pathway also involves Dishevelled (Sheldahl et al., 2003). Signalling through this pathway leads to the activation of two calcium-sensitive proteins, Protein Kinase C and Calcium/Calmodulin-dependent Kinase II (Kuhl, 2004). Additionally, intracellular calcium increases that are induced by non-canonical Wnts through Frizzled are dependent on the activity of heterotrimeric G proteins, acting upstream of Dishevelled. Finally, there is some evidence that signalling through this pathway may antagonise canonical Wnt signals (Kuhl, 2004; Veeman et al., 2003). It is of note that, although the PCP/convergent extension pathway and the Wnt/calcium pathway are often described as separate pathways, they share many components, including the same Wnt ligands, as well as requiring the same domain of Dishevelled for their activity. Moreover, activation of the c-Jun N-terminal Kinase (JNK) pathway occurs downstream of non-canonical roles of Dishevelled and Frizzled, as well as Rho-family GTPase signalling and activation of Protein Kinase C (Fanto et al., 2000; Moriguchi et al., 1999; Pandur et al., 2002; Weber et al., 2000; Yamanaka et al., 2002). Nevertheless, it is possible that the pathways diverge downstream of Prickle and Dishevelled, and that different effector branches of non-canonical Wnt signalling exist (reviewed in Veeman et al., 2003).

Developmental roles of Wnt signalling.

Planar polarity and convergent extension.

Non-canonical Wnt signalling was first identified for its roles in planar polarity in the wing hairs, body bristles and ommatidia in the eye of *Drosophila*, and in regulating certain gastrulation movements. Planar cell polarity describes the properties of some epithelia, which are polarised along an axis perpendicular to the apical-basal axis. The best characterised system of PCP is that of the orientation of actin-rich bundles which give rise to hairs called trichomes (reviewed in Fanto and McNeill, 2004). Each cell in the wing produces one trichome, pointing towards the distal end of the wing. Many PCP mutations have been found which affect either the orientation, sub-cellular localisation or number of hairs produced. The precise mechanism by which polarity is established is not known, but it is likely that it is maintained and stabilised by localised interactions between cells. Frizzled and Dishevelled localise to the distal membrane of cells, while Prickle and Strabismus are found at the proximal membrane, and interactions between proteins at the distal membrane of one cell with those at the proximal membrane of its neighbour stabilise this system (Fanto and McNeill, 2004; Mlodzik, 2002). In the eye, PCP involves the orientation of ommatidia, that undergo rotation in different directions, depending on their localisation relative to the equator of the eye, and the differentiation of two photoreceptor cells, R3 and R4 (Fanto and McNeill, 2004). It is thought that Frizzled is differentially activated in the R3 and R4 precursors, which leads to differential activation of the Notch signalling pathway between these two cells.

Thus, in this instance, the establishment of PCP leads to transcriptional activity and changes in cell identity. It is also interesting to note that, in the eye, PCP specifies the orientation of groups of cells, rather than the polarity of single cells, as occurs in the wing. Additional genes are involved in setting up the initial polarity both in eye and wing, such as *fat* and *dachsous*, encoding atypical cadherins. These genes appear to act upstream of Frizzled and are expressed in opposing gradients, both in the wing and in the eye, but the way in which they are regulated is not clear (Fanto and McNeill, 2004; Mlodzik, 2002). Currently, no single upstream gene is known to regulate overall polarity in these tissues, and many models involve a combination of a localised graded signal, defining and setting up the axis of polarity, and local cell-cell interactions, which maintain coherence within the organisation of the tissue (see for example Ma et al., 2003).

In vertebrates, planar cell polarity is observed in the cochlear hair cells, whose actin-rich bundles of hairs, called stereocilia, form organised arrays, with the apex pointing in the same direction. Recent work has shown that certain genes of the PCP pathway affect this organisation, particularly Wnts and Wnt inhibitors, and homologues of *strabismus* and *flamingo* (Curtin et al., 2003; Dabdoub et al., 2003; Montcouquiol et al., 2003). The best characterised occurrence of non-canonical Wnt signalling is the control of convergent extension movements during gastrulation. These movements involve the accumulation of cells on the dorsal side of the embryo, and the elongation of the anterior-posterior axis, which is achieved by intercalation of cells (Heisenberg and Tada, 2002; Tada et al., 2002). Unlike PCP, where cells have a defined polarity, convergent extension requires that cells adopt a bipolar morphology along the medio-lateral axis (Veeman et al., 2003). Nevertheless, work in

Xenopus and, more recently, genetic tools in the zebrafish have uncovered a role for non-canonical Wnt signalling in regulating this polarisation and consequent cell movements (Du et al., 1995; Heisenberg et al., 2000; Moon et al., 1993). Genes recovered from genetic screens for disruption of convergent extension in the zebrafish have been found to encode two Wnt ligands (*silberblick*/wnt11 and *pipetail*/wnt5, Heisenberg et al., 2000; Rauch et al., 1997), as well as homologues of *strabismus* (called *trilobite*, Jessen et al., 2002). Knockdown and overexpression of a *prickle* homologue shows that, as in the *Drosophila* PCP pathway, it is also involved in regulating convergent extension (Carreira-Barbosa et al., 2003). It is interesting that, although the PCP pathway in vertebrates involves Wnts as ligands for Frizzled, it does not appear to require that these Wnts form gradients, indicating that it is likely that they only act permissively (Heisenberg et al., 2000). Finally, two genes of the convergent extension pathway, *trilobite* and *prickle1*, have been involved in the tangential migration of branchiomotor neurons, particularly of the facial nerve (nVII), which normally migrate from r4 to r6 and r7 (Bingham et al., 2002; Carreira-Barbosa et al., 2003).

Thus, non-canonical Wnt pathways control polarity and movement in many different systems, but, in most cases, do not appear to affect gene expression or cell type patterning. This is quite distinct from canonical Wnt signalling, which has many important roles in patterning during embryogenesis.

Patterning functions.

Although one of the first and most striking phenotypes of Wnt overexpression to be observed was the induction of secondary axes in *Xenopus*

embryos (McMahon and Moon, 1989), subsequent work has shown that Wnts themselves are not involved in axis formation. Instead, a mechanism occurring during cortical rotation causes β-Catenin to localise preferentially in the future dorsal side of the embryo, thus inducing the two early organisers which are responsible for dorsal axis induction (for a recent review, see De Robertis and Kuroda, 2004). However, recent work has found a role for Wnt signalling in regulating anterior-posterior patterning at subsequent stages in development. Wnts are found posteriorly, and Wnt inhibitors are expressed anteriorly. The activation of Wnt signalling inhibits the proper formation of anterior structures, such as occurs in the zebrafish *headless* mutant. In this case, *tcf3* is mutated, and the phenotype is due to de-repression of Wnt target genes (Kim et al., 2000). Thus, the posterior-to-anterior gradient of Wnts is translated into an anterior-to-posterior gradient of repression of Wnt targets, leading to discrete domains, such as forebrain or midbrain, being established (Dorsky et al., 2003; Kiecker and Niehrs, 2001; Nordstrom et al., 2002).

The *Drosophila* Wnt family member, *wingless*, is one of the better characterised genes of the family. Among its numerous roles, the patterning of parasegments has been presented above. Studies of the role of Wingless in patterning the wing imaginal disc have suggested that it may act as a morphogen during development (reviewed in Strigini and Cohen, 1999). Wingless acts at a distance from its source to induce at least three genes in nested patterns : *achaete*, *distal-less* and *vestigial* requiring gradually lower levels of Wingless protein (Neumann and Cohen, 1997).

The vertebrate homologue of *wingless*, *wnt1*, has important roles in the formation and maintenance of the midbrain. During development, *wnt1* is

transiently expressed throughout the midbrain, and is then restricted to the midbrain-hindbrain boundary and the roof plate of the midbrain (Wilkinson et al., 1987). In *wnt1* knockout mice, the whole midbrain and anterior hindbrain are deleted (Mastick et al., 1996; McMahon and Bradley, 1990). However, the midbrain is specified in these mutants, but is gradually deleted, indicating that Wnt1 controls either the maintenance of midbrain fate, or proliferation and/or apoptosis (McMahon et al., 1992). The precise roles of Wnt1 in the midbrain and at the midbrain-hindbrain boundary have not yet been elucidated, but strong evidence points towards a specific role in the regulation of proliferation, as overexpressing Wnt1 has no discernible effect on midbrain gene expression (Panhuysen et al., 2004).

Regulation of proliferation.

The regulation of cell proliferation is a recurring theme for Wnt/β-Catenin signalling. Several studies in the vertebrate neural tube, in which either Wnt1 or β-Catenin is overexpressed, have shown that ectopic cell proliferation occurs, at the level of the spinal cord, midbrain or forebrain (Chenn and Walsh, 2002; Dickinson et al., 1994; Panhuysen et al., 2004). The primary mode of action of Wnt signalling leading to an increase in cell number is to bias the choice cells make between differentiation and re-entry into the cell cycle towards the latter alternative (Chenn and Walsh, 2002; Megason and McMahon, 2002). It is also known that Wnt1 directly regulates *cyclind1* expression, which is thought to mediate at least in part its mitogenic effect (Shtutman et al., 1999; Tetsu and McCormick, 1999). In the spinal cord, this has led to a model in which the dorsal midline, which is a source of two mitogenic Wnts, Wnt1 and Wnt3a, regulates

the growth of the neural tube in the dorso-ventral axis, and is responsible for the ventral-to-dorsal order of neuronal differentiation (Megason and McMahon, 2002). Wingless also has a role in regulating cell number in the wing imaginal disc in *Drosophila*, although it appears that it only indirectly regulates proliferation, and has an important role in inhibiting apoptosis (Giraldez and Cohen, 2003).

The link between Wnt/β-Catenin signalling and cell proliferation is of particular interest as several lines of evidence point to roles of this pathway, when deregulated, in cancer (reviewed in Giles et al., 2003). Approximately 90% of colorectal cancers have activating mutations in the Wnt pathway, although few of these actually involve Wnt ligands. Loss-of-function mutations of APC are involved in many cases, and it appears that even a heterozygous loss of APC can result in increased risk of colorectal cancer, as well as a rare dominant inherited disease, familial adenomatous polyposis, where patients develop multiple adenomas in the colorectum (Groden et al., 1991; Nakamura et al., 1991). Conversely, gain-of-function mutations of β-Catenin have been implicated in many cancer types. Most β-Catenin mutations affect the phosphorylation sites for GSK3β, such that the mutant protein is not targeted for degradation (Polakis, 1999). However, Wnt/β-Catenin signalling occurs normally in adults, to regulate stem cell proliferation and differentiation in the intestine and colon, implying that progression to cancer involves several changes additionally to activating Wnt signalling (Giles et al., 2003).

Aims of this study.

I set out to understand how zebrafish hindbrain boundary cell formation is regulated. I also decided to investigate possible relationships that boundary cells may have with the anterior-posterior organisation of neural cell types within each rhombomere. Zebrafish hindbrain boundaries express *wnt1*, which is a candidate to mediate possible signalling roles of boundaries. In this thesis, I will first describe the normal pattern of neurogenesis, neural cell type organisation and boundary cell marker expression in the zebrafish hindbrain, and then analysis of the function of Wnt1 in loss and gain of function experiments.

Materials and methods.

Fish maintenance.

Adult zebrafish were maintained in 10h night/14h day cycles. Wild-type zebrafish embryos were obtained by natural spawning and raised at 28°C, as described (Westerfield, 1993). For stages earlier than 24h, embryos were raised at 22°C and staged by somite number. Control and injected embryos were stage-matched by somite number or eye pigmentation (Kimmel et al., 1995). 24h-old *after eight* embryos, mutant for *deltaD*, were obtained fixed from Phil Taylor at Cancer Research (Lincoln's Inn Fields). Embryos were manually dechorionated after fixation.

Morpholino oligonucleotide and RNA injections.

Injection protocol.

0.5-2.5 pmol of morpholino oligonucleotide or 0.2-1ng of capped RNA was injected into 1-4 cell blastomeres, or into one cell at the two to sixteen cell stage to obtain mosaic RNA expression, using an electric microinjector (Inject Matic, Switzerland). In the case of embryos injected with both wnt1MO and stabilised β-catenin, the morpholino was injected at the one cell stage, and β-catenin RNA was injected into one cell at the eight cell stage, together with membrane-targeted GFP RNA. The needles used for injection were obtained from 1.0mm external diameter glass capillaries with internal filament (Harvard Apparatus, Kent) with a David Kopf Instruments needle puller.

Morpholino oligonucleotides.

Morpholino oligonucleotides (MOs) were purchased from Gene Tools, LLC (Oregon). MOs were kept as 1mM stock solutions by addition of pure water (Sigma), and further dilutions of between 1:5-1:20 were made in pure water for injection. The following MO sequences were used :

wnt1 MO: AGCAACGCGAGAACCCGCATGATAT

asha MO: CCATCTTGGCGGTGATGTCCATTTC

ashb MO: TCGTAGCGACGACAGTTGCCTCCAT

ngn1 MO: ATACGATCTCCATTGTTGATAACCT

deltaA MO: CTTCTCTTTTCGCCGACTGATTCAT

rfng MO: as described (Cheng et al., 2004)

tcf3b MO: as described (Dorsky et al., 2003)

standard control MO : CCTCTTACCTCAGTTACAATTTATA.

RNA constructs.

pCS2 plasmids containing inserts encoding full-length chick Wnt1 (cWnt1), stabilised β-catenin, lacking 87 amino acids that include the phosphorylation site for GSK3 (Domingos et al., 2001, both gifts of Nobue Itasaki), membrane-targeted GFP (Moriyoshi et al., 1996) or nuclear-localised GFP with normal or mutated wnt1MO and rfngMO recognition sequences (described below) were linearised with NotI. In order to obtain capped mRNAs, the following reagents were incubated at 37°C for 2h :

2μL linearised plasmid at 1μg/μL

20μL transcription buffer

12.5μL DTT

10μL nucleotide stock (10mM ATP, CTP, UTP, 1mM GTP, Pharmacia Biotech)

10μL methylated cap (Roche)

2μL RNasin (Promega)

2μL SP6 RNA polymerase (Roche)

41.5μL pure water (Sigma)

After incubation, 2μL of RQ1 DNase (Promega) were added for 20 minutes, then the reaction was purified using Microspin G-50 columns (Amerham), and stored at -80°C after 1μL was run on a gel for verification.

In situ *hybridisation.*

Protocol.

In situ hybridisation was carried out as described in Xu *et al.* (1994), with the following modifications. No proteinase K treatment was carried out, consequently, embryos were immediately pre-hybridised after rehydration. All steps of pre-hybridisation, hybridisation and washes in 50% formamide-2X SSC, 2X SSC and 0.2X SSC were carried out at 68°C. The anti-DIG-AP antibody (1:2000, Roche) was not preabsorbed. After the colour reaction, embryos were briefly fixed in 4% paraformaldehyde, then rinsed in PBS-0.1% Tween20 (PBST) and stored and flat-mounted in 70% glycerol in PBST. Alternatively, after fixing, embryos were treated for EphA4 or Hu immunohistochemistry (see below). In the case of two-colour *in situ* hybridisation, both DIG- and fluorescein-labelled probes were hybridised simultaneously. The fluorescein-labelled probe was detected first, using anti-fluorescein-AP (Roche) and Fast Red tablets (Roche) for the colour reaction. Once this reaction was carried out,

the alkaline phosphatase was inactivated by incubating the embryos twice in 0.1M glycine/HCl for 15 minutes. Embryos were then briefly fixed in 4% paraformaldehyde, then rinsed in PBS and treated with anti-DIG-AP antibody and colour as described above.

Probes used.

The following probes have been previously described :

rfng (Cheng et al., 2004; Qiu et al., 2004)

wnt1 (Molven et al., 1991)

foxb1.2 (Moens et al., 1996)

asha and *ashb* (Allende and Weinberg, 1994)

ngn1 (Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998)

pax6 (Krauss et al., 1991)

wnt8b (Kelly et al., 1995)

netrin1a (Lauderdale et al., 1997)

crapb2 (Sharma et al., 2003)

deltaA, *deltaB* and *deltaD* (Haddon et al., 1998)

$p27^{Xic1}$-*a* (obtained from the RZPD, Geling et al., 2003)

tbx20 (Ahn et al., 2000)

olig2 (Park et al., 2002)

pax3 (Seo et al., 1998)

dbx1a (Fjose et al., 1994)

evx1 (Thaeron et al., 2000)

krox20 (Oxtoby and Jowett, 1993)

hoxb1a (Prince et al., 1998)

In the case of *gfap* and *cyclinD1*, the genes have been cloned (Nielsen and Jorgensen, 2003; Yarden et al., 1995, respectively), but the expression patterns have not been described. For both of these genes, I obtained ESTs from the RZPD (Berlin, Germany) corresponding to the published sequences, which I used to make probe from (ESTs : fc83a12.y1 for *cyclinD1* and fc66a07.y1 for *gfap*, both of which were linearised with SalI and transcribed with SP6 for antisense RNA). *her9* is a new hairy/enhancer of split family member identified in this lab by Andrea Pasini, which will be the subject of a descriptive paper.

Immunohistochemistry.

Immunohistochemistry was carried out with the following antibodies : anti-EphA4 (1:500, Irving et al., 1996b), anti-HuC/HuD (1:200, Molecular Probes), anti-GFAP (1:500, Sigma), RMO-44 (anti-NF-M, 1:25, Zymed) and anti-phosphorylated histone H3 (1:200, Upstate). The secondary antibodies used were Alexa Fluor 488, 594 or 647 goat anti-rabbit or goat anti-mouse (all 1:500, Molecular Probes), or HRP-goat anti-rabbit (1:250, DAKO). For EphA4 and Hu immunohistochemistry, embryos fixed in 4% PFA were blocked in PBST containing 5% goat serum for at least 1h, then incubated overnight at 4°C in the antibody diluted in PBST with 2% goat serum. After several washes in PBST, the embryos were incubated overnight at 4°C (or 2h at room temperature) in the

secondary antibodies diluted in PBST with 2% goat serum, then washed in PBST and stored and flat-mounted in 70% glycerol in PBS or in Vectorshield medium (Vector) in the case of fluorescent secondaries. For GFAP, embryos were fixed 40 minutes at -20°C in 90% ethanol, 10% acetic acid, then treated as normal. In the case of phospho-histone H3, PBS containing 0.1% Triton X-100 was used instead of PBST. For DAB colour reaction, embryos were incubated in a solution of 1mg/mL DAB, 0.02% hydrogen peroxide in PBS until precipitate formed. For RMO-44 immunohistochemistry, embryos were fixed in 2% trichloroacetic acid for 4h at room temperature, washed in PBST twice, then in distilled water and incubated in acetone for 10 minutes at -20°C, and again rinsed in distilled water then PBST several times. After this, embryos were processed as for other antibodies.

Molecular biology.

pCS2+ constructs encoding nuclear localised GFP (nlsGFP) with wnt1MO or rfngMO recognition sequences were generated as follows. The nuclear localised GFP was excised from pUAS-GFPnls (gift of Cyrille Alexandre, Vincent lab) and directionally cloned into pCS2+ using XhoI and XbaI restriction enzymes. This construct was then used to generate the recognition sequence constructs by excising the start codon (located between XhoI and BglIII restriction sites) and purifying this digest. Oligonucleotides containing the morpholino recognition site, XhoI and BglIII overhangs and an inserted NotI site for verification purposes were pre-annealed by incubating 1μg of forward and reverse oligonucleotide in 20μL total volume for 4 minutes at

98°C, then ten minutes each at 90°C, 80°C, 70°C and 60°C. These were then ligated into the linearised nlsGFP-pCS2+ plasmid and transformed. The insertions were verified by sequencing, as well as restriction analysis of NotI, as a site was inserted, and HindIII, as its restriction site was removed in this process. The following oligonucleotides were used (morpholino recognition sequences are shown in capital letters, start codons underlined, and mutated nucleotides in bold) :

rfngMO : tcgaggcggccgcATGCACTTATCCCATGTCGCCTCCAata

gatctatTGGAGGCGACATGGGATAAGTGCATgcggccgcc

mutated rfngMO : tcgaggcggccgcATGCA**T**TT**G**TC**G**CA**C**GT**G**GCCTCCAata

gatctatTGGAGGC**C**AC**G**TG**C**GA**C**AA**A**TGCATgcggccgcc

wnt1MO : tcgaggcggccgcATATCATGCGGGTTCTCGCGTTGCTca

gatctgAGCAACGCGAGAACCCGCATGATATgcggccgcc

mutated wnt1MO : tcgaggcggccgcATAT**G**ATGCG**C**GT**A**CT**G**GC**A**TTGCTca

gatctgAGCAA**T**GC**C**AG**T**AC**G**CGCAT**C**ATATgcggccgcc

Characterisation of neurogenesis and boundary formation in wild-type embryos.

Results.

Patterns of neuronal differentiation in the zebrafish hindbrain.

Previous studies of the organisation of neurons and glia in the zebrafish hindbrain have uncovered a stereotypical pattern along the anterior-posterior axis within each rhombomere (Fig.4B, Hanneman et al., 1988; Trevarrow et al., 1990). However these studies have been limited by few molecular markers being available at the time they were carried out. In order to gain a better understanding of the timings and locations of neurogenesis and gliogenesis in the hindbrain, I analysed a series of markers by *in situ* hybridisation and immunohistochemistry. In most cases, double labelling was carried out with EphA4 antibody (Irving et al., 1996b), which labels rhombomeres 3 and 5 (r3 and r5), to determine in which rhombomere the staining occurs, and where within that rhombomere, relative to its interfaces with adjacent segments.

Localisation of post-mitotic neurons.

To analyse the global pattern of neurogenesis, I have used an antibody against the RNA-binding proteins HuC and HuD, which labels all post-mitotic neurons (Park et al., 2000a; Park et al., 2000b; Wakamatsu and Weston, 1997).

The earliest neurons detected, as early as the 10 somite stage (14h of development, Fig.6A), are located at the r2/r3 boundary and the r4/r5 boundary, as well as in the centre of r4, and two neurons in the centre of r6. The cell in the centre of r4 presumably corresponds to the Mauthner cell, which is the earliest-born neuron (Mendelson, 1986).

By 16.5 hours of development (15 somites, Fig.6B), there are more neurons, but they are still individually identifiable. Compared to the 10 somite stage, there are new neurons in r2, near to the r1/r2 interface, as well as adjacent to the r2/r3 interface. There are also neurons in segments where none could be seen at 10 somites, in the centre of r3 and of r5, as well as two more neurons in each of r4 and r6.

At 24h, the distribution of HuC/HuD staining shows that neurons are more numerous, forming clusters in the centre of each rhombomere, and that little differentiation has occurred at the interfaces between segments (Fig.6C). However, by 30h (Fig.6D), a pattern has been established which persists until at least 48h (not shown). A single row of neurons occupies the segment interfaces, separated from the large neuronal clusters in the centres of the rhombomeres by a row of Hu-negative cells. This distribution corresponds to the pattern which has been described previously (Hanneman et al., 1988; Trevarrow et al., 1990).

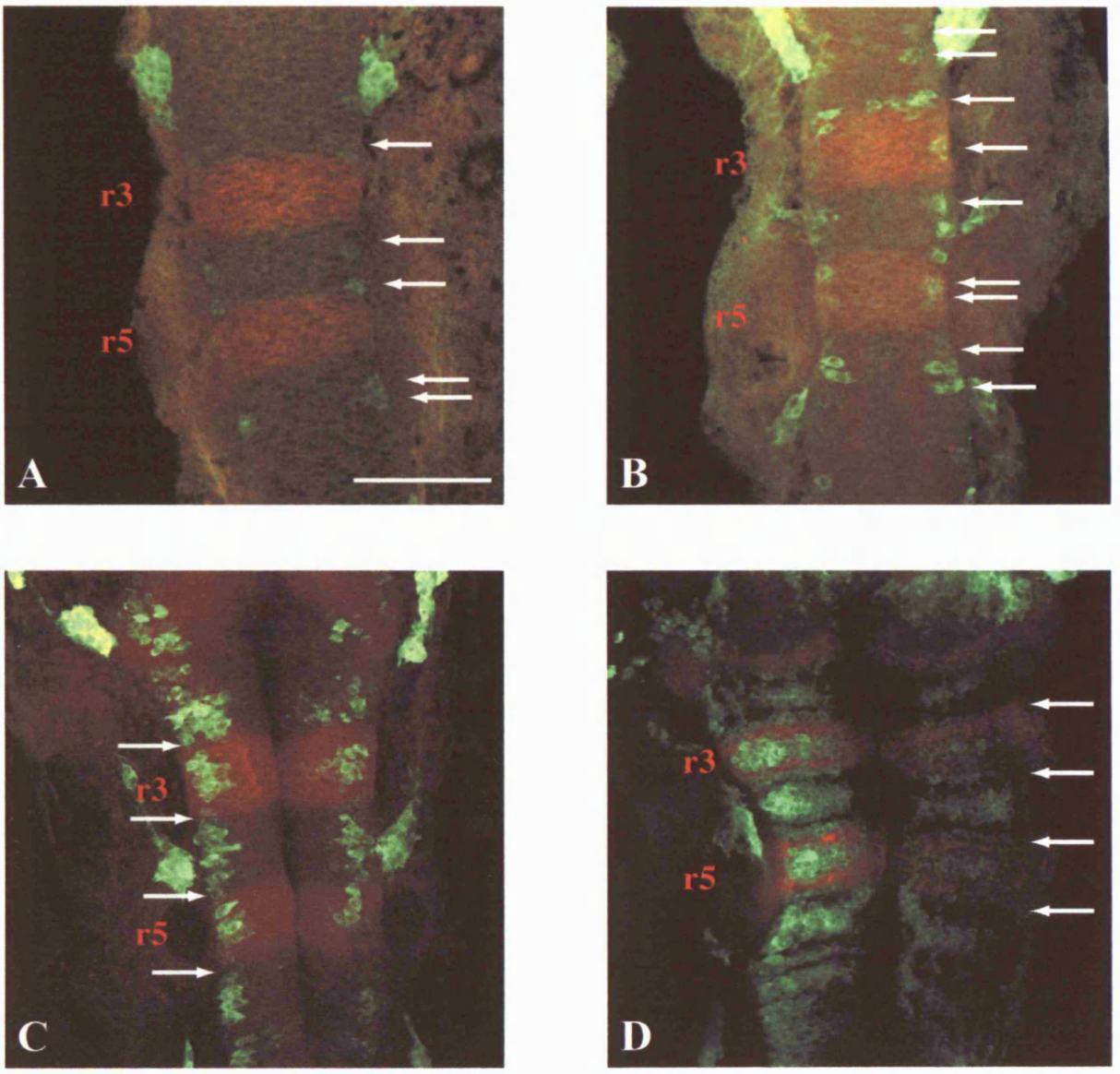


Fig. 6 : Localisation of post-mitotic neurons.

HuC/HuD immunohistochemistry (green), with EphA4 (red) labelling rhombomeres 3 and 5. Dorsal views of flat-mounted embryos, anterior to the top, A-C are maximum value projections of confocal stacks, showing the strongest labelling at all "z" locations, D is a single confocal section. A : 10 somite stage, showing a few Hu-positive cells, at the r2/r3 and r4/r5 boundaries, and in the centre of r4 and r6 (arrows). At the 15 somite stage (B), additional Hu-positive cells are shown with arrows. C : 24h embryo, showing most additional Hu-positive cells in the middle of rhombomeres (arrows show boundaries, where less staining is seen). D : 30h embryo, displaying a distinct pattern at rhombomere boundaries (arrowed) of a single Hu-positive row of cells surrounded by a single row of Hu-negative cells (presumptive glia). Scale bar : 50μm. r3 : rhombomere 3, r5 : rhombomere 5.

Thus, until at least 15 somites, individual neurons are identifiable at the centre and boundaries of rhombomeres. By 24h, neurons are present mainly in clusters at the centre of each rhombomere, and later still, there are neurons at boundaries, separated from the large neuronal clusters in the rhombomere centres by a row of non-neuronal cells.

Analysis of neuronal differentiation between 18h and 48h.

The arrangement of post-mitotic neurons is not uniform throughout the hindbrain. Looking only at stages when significant numbers of neurons are present, especially at 24h, it is apparent that neurons are concentrated in some areas (rhombomere centres) and present in small numbers in others (boundary regions). This suggests that differentiation is regulated along the anterior-posterior axis within each rhombomere. A better picture of the neurogenesis process would therefore be obtained by analysing where neuronal differentiation takes place in the zebrafish hindbrain.

I have therefore analysed the expression patterns of several classes of genes involved in the process of neurogenesis. First, proneural genes, which encode bHLH transcription factors involved in the selection of neuronal progenitors from undifferentiated neuroepithelium, leading to the neuronal differentiation programme (reviewed in Bertrand et al., 2002). The zebrafish has three known proneural genes, *asha*, *ashb*, and *ngn1* (Allende and Weinberg, 1994; Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998). Second, Delta genes, which are targets of proneural genes in all species analysed (Casarosa et al., 1999; Haenlin et al., 1994; Hans and Campos-Ortega, 2002; Heitzler et al.,

1996; Kunisch et al., 1994; Ma et al., 1998). These genes are involved in selecting the cell that will differentiate by lateral inhibition of differentiation in neighbouring cells. In zebrafish, there are four known Delta genes, but *deltaC* is expressed in few cells in the central nervous system (Yi-Chuan Cheng, unpublished data, and Haddon et al., 1998). Moreover, *deltaA* and *deltaD* are expressed broadly in proneural patches, but have higher expression levels in prospective neurons, while *deltaB* transcripts are found only in the differentiating neurons (Haddon et al., 1998). The third type of marker I have used is the cdk inhibitor $p27^{Xic1}$-*a*, which promotes cell cycle exit and is required for neuronal differentiation (Carruthers et al., 2003; Geling et al., 2003; Ohnuma et al., 1999).

At 18h of development, *asha* and *ngn1* are expressed in the dorsal and ventral half of each rhombomere, respectively, but are not expressed in boundary cells (Fig.7A,C). *ashb* transcripts are detected at high levels in r4 (Fig.7B). *deltaA* and *deltaD* are also expressed throughout the hindbrain, except rhombomere boundaries (Fig.7D,F). However, while these patterns suggest that differentiation is occurring throughout rhombomeric segments, expression of $p27^{Xic1}$-*a* and of *deltaB* shows that most differentiation at this stage is actually located ventrally in the centre of each segment (Fig.7E,G).

By 26h, the expression of *ashb* and *ngn1* proneural genes now occurs in stripes adjacent to rhombomere boundaries, with weaker expression maintained throughout the rest of the ventricular zone, except at boundaries (Fig.7I,J). That the stronger expression corresponds to presumptive neuroblasts is confirmed by the fact that *deltaA*, *deltaB* and *deltaD*, as well as $p27^{Xic1}$-*a* are all expressed in similar stripes adjacent to rhombomere boundaries (Fig.7K-N). *asha* expression at this stage is limited to scattered cells but remains excluded from boundaries

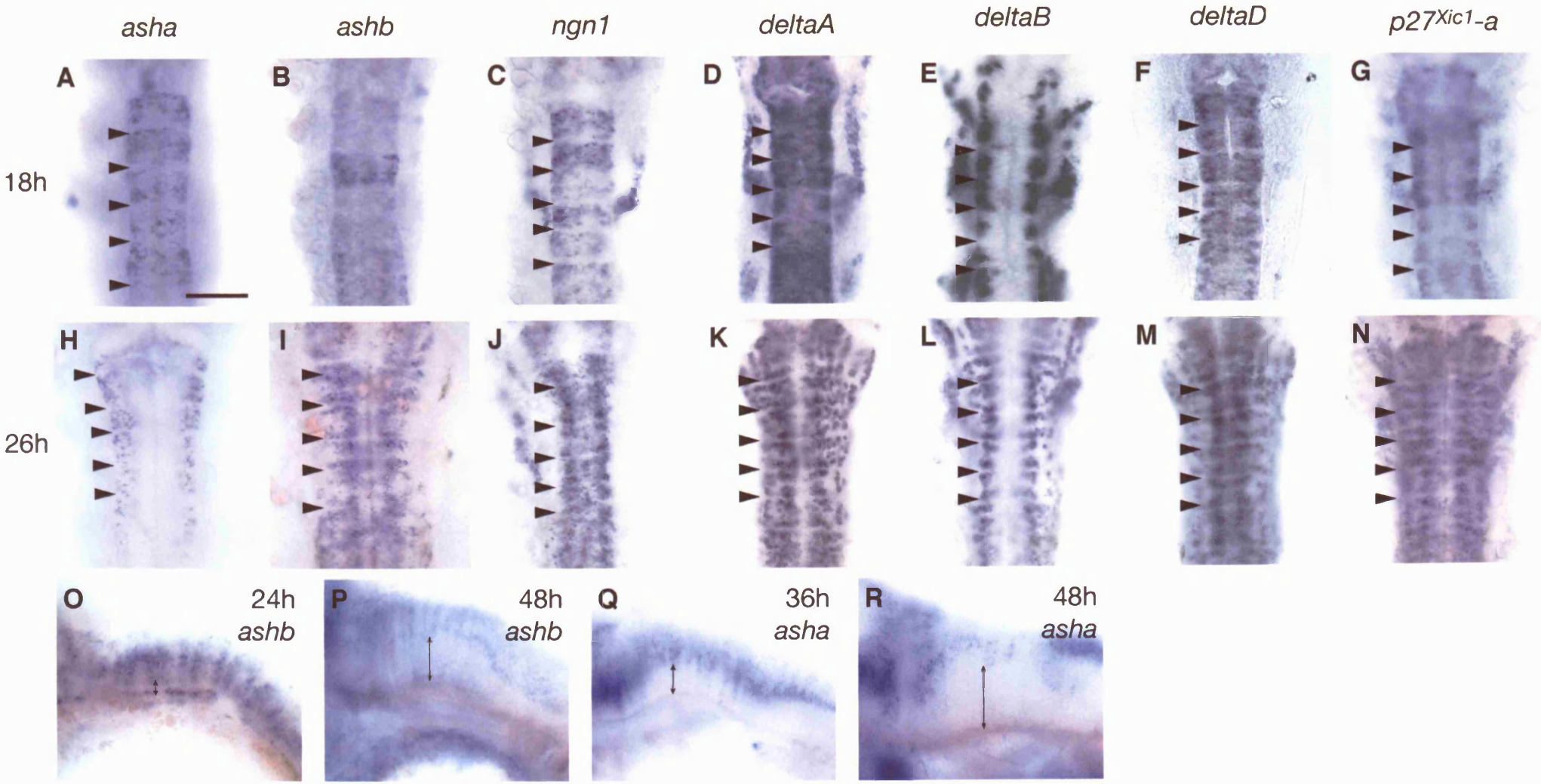
(Fig.7H), but from approximately 30h onwards it is also found in stripes flanking boundaries. The hindbrain is also patterned in the dorso-ventral axis, in that *ngn1* is expressed more ventrally, *ashb* medially and *asha* dorsally.

Finally, between 26h and 48h it can be seen that differentiation occurs in a dorsal to ventral manner, as the ventral-most expression of *asha* and *ashb* recedes dorsally over this time frame (Fig.7O-R). This is also evident from the difference in the timing of the appearance of *dbx1a* (medial) and *pax3* (dorsal) stripes (see below, Fig.8E,I).

The expression patterns of these genes reveal that differentiation is localised in the hindbrain : while at 18h, it occurs mainly in the centre of each rhombomere, by 24h, stripes of differentiation are present adjacent to the boundaries. The latter pattern persists until at least 48h, but shifts dorsally over time.

Fig. 7 : Neuronal differentiation in the zebrafish hindbrain.

Expression of proneural and delta genes, and of the cell cycle inhibitor $p27^{xic1}$-a at 18h (A-G), 24h (H-O), 36h (Q) and 48h (P,R). A-N are dorsal views, with anterior at the top, O-R are lateral views, with anterior to the left. All markers shown display gaps of expression at rhombomere boundaries, indicated by arrowheads. While most markers are segmental at 18h (A-D, F), differentiation occurs in the centre of rhombomeres (E,G), but at later stages, it takes place mainly in stripes adjacent to rhombomere boundaries (I-R), in a ventral to dorsal wave (O-R, arrows indicate that the distance between the floor plate and the most ventral labelled cells increases over time). Scale bar : 100μm.


asha
ashb
ngn1
deltaA
deltaB
deltaD
p27Xic1-a
A
B
C
D
E
F
G
18h
H
I
J
K
L
M
N
26h
O
24h
ashb
P
48h
ashb
Q
36h
asha
R
48h
asha

Localisation of selected individual neuronal and glial cell types.

A more detailed picture of neurogenesis and gliogenesis is gained by examining the distribution of specific cell types within the hindbrain, which may help to refine the previous observations of anterior-posterior pattern within each segment, particularly as the differentiation patterns show important temporal differences. I have therefore analysed markers for cell types that differentiate at various time points.

Reticulospinal neurons are the earliest to develop, (with only four exceptions, all are born between 7.5 and 15 hours of development Hanneman et al., 1988; Mendelson, 1986). The RMO-44 antibody, which recognises neurofilament-M, labels a subset of these reticulospinal neurons, which are found in the centre of each rhombomere (Fig.8A). Cranial motor neurons are also born relatively early, detectable between 16-20 hours post fertilisation (Chandrasekhar et al., 1997). *In situ* hybridisation with the T-box transcription factor *tbx20* as a marker, shows that these neurons are present in clusters in the centre of each rhombomere at 48h (Fig.8B), although they initially appear as an unsegmented column of neurons (Ahn et al., 2000; Chandrasekhar et al., 1997).

dbx1a labels progenitors and neurons at medial locations along the dorso-ventral axis (Fjose et al., 1994). Ventricular labelling forms a continuous column along the anterior-posterior axis, but from about 24h (Fig.8E), neurons are present in rows of cells adjacent to rhombomere boundaries. This pattern is similar at 48h (Fig.8F,G), when two clear stripes are visible in each rhombomere. Transverse cryostat sections of *dbx1a*-labelled embryos at 48h (Fig.8H) show that most labelled cells are in the post-mitotic mantle zone (MZ), at only one dorso-ventral location.

A more dorsal marker, *pax3*, is initially expressed continuously along the dorsal ventricular zone (Fig.8I). By 48h, transcripts are detected in bilateral stripes adjacent to boundaries (Fig.8J,K). As the neural tube at the level of the hindbrain has a ventricular zone shaped like a "T" at this stage (Lyons et al., 2003), the dorsal location of these cells is visible as a lateral position (compare for instance to the more ventral *dbx1a* pattern, which is present medially relative to *pax3* expression).

Finally, oligodendrocyte progenitors first differentiate around 48h. *olig2* labels both motor neuron progenitors and oligodendrocyte progenitors in the spinal cord (Park et al., 2002), but in the hindbrain, no expression can be detected until 48h (not shown), indicating that it is not expressed in motor neurons. At 48h, *olig2* expression can be detected ventrally in the centre of each rhombomere (Fig.8C,D).

The analysis of markers for distinct neuronal subtypes reveals that they have stereotypical localisations both along the anterior-posterior and dorso-ventral axes. As a general rule, early-born neurons are found in rhombomere centres, whereas later born neurons form stripes adjacent to boundaries. Oligodendrocyte progenitors are born in the centre of rhombomeres at two days of development.

Fig. 8 : Spatial distribution of selected cell types in the hindbrain.

Immunohistochemistry (A) and *in situ* hybridisation (B-K) of whole-mount embryos (A-G, I-K) and cryostat transverse sections (H) for the indicated marker and stage. All views are dorsal with anterior to the top. A: Reticulospinal neurons, labelled with RMO-44, are found in the centre of rhombomeres. B: Distribution of cranial motor neurons, mostly in the centre of rhombomeres. Oligodendrocyte progenitors are also found ventrally in rhombomere centres (C,D). E-H : stripes of neuronal *dbx1a* expression are found outside the ventricular zone from 24h. Ventricular zone (VZ) and post-mitotic mantle zone (MZ) are indicated on H and were determined by a combination of DAPI stain and immunohistochemistry with the pan-neuronal marker Hu. Stripes of *pax3* expression are also seen at 48h (J,K), but not at 24h (I). Scale bar : 50μm in A and H, 100μm in B-G and I-K.

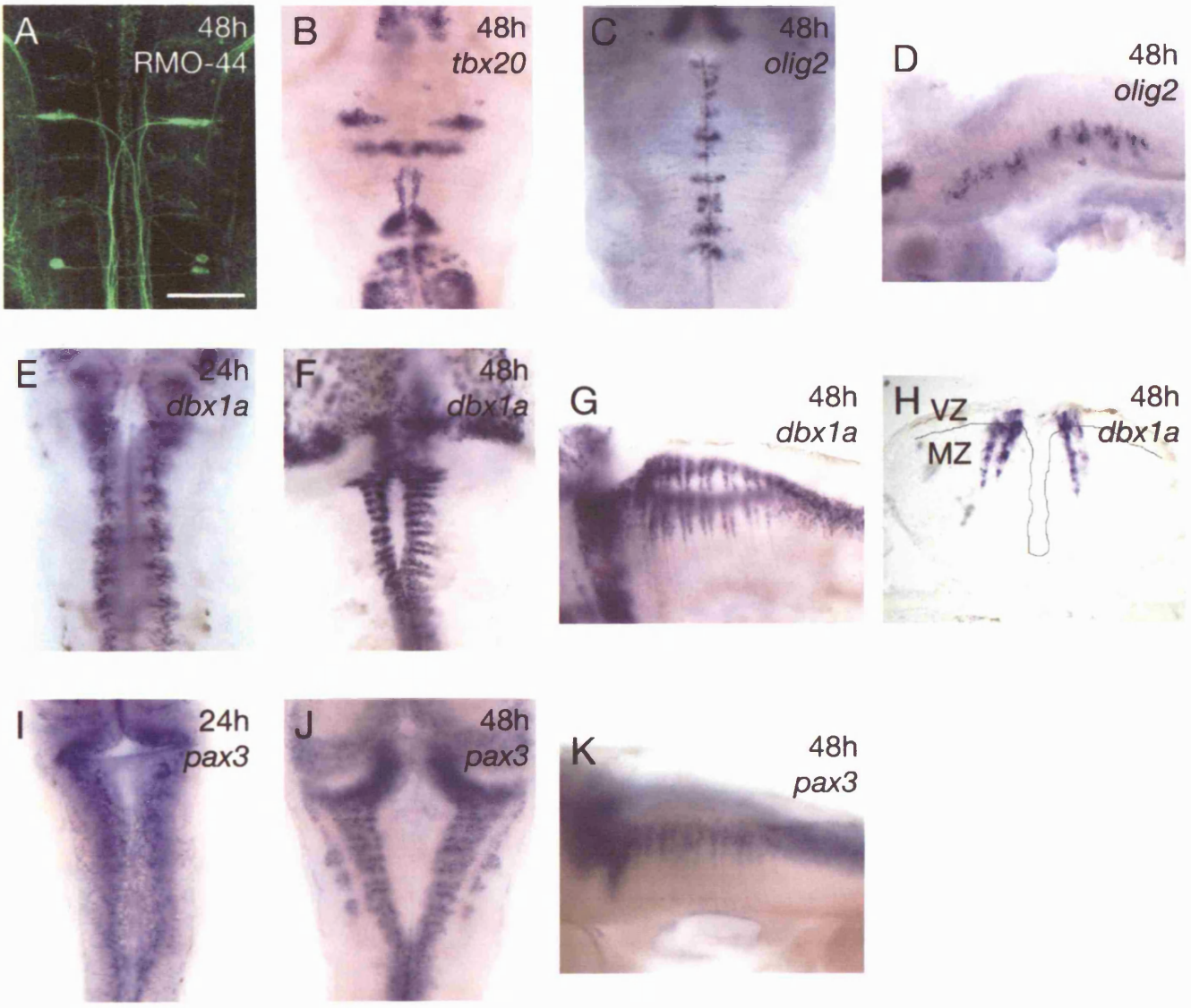

A
48h
RMO-44
B
48h
tbx20
C
48h
olig2
D
48h
olig2
E
24h
dbx1a
F
48h
dbx1a
G
48h
dbx1a
H
VZ
MZ
48h
dbx1a
I
24h
pax3
J
48h
pax3
K
48h
pax3

Fig. 9 : Differentiation at interrhombomeric interfaces.

Expression of *evx1* (A-D) and of *gfap* (E-G) and immunohistochemistry against the GFAP antigen (H). All views are dorsal with anterior at the top. A : 18h embryo, focussed on a dorsal plane, showing labelled cells at both boundaries of rhombomere 4 (r4, boundaries shown with arrowheads). B,C : medially- and dorsally-focussed view of the same 24h embryo, showing the presence of two different populations of *evx1*-positive cells, one of which is found at rhombomere boundaries (arrowheads in C). By 26h, neurons at boundaries (arrowheads) form continuous columns. E : expression of *gfap* at 18h, with stronger expression in rhombomeres 4 and 6, while at 24h (F), expression is more uniform in the ventricular zone, although slightly stronger in r4, and upregulated in rhombomere boundaries. The boundary expression domain splits into two (arrow in F). G shows a false-coloured *in situ* hybridisation against *gfap* in red, followed by immunohistochemistry against HuC/HuD (green), to confirm that the gaps in Hu labelling correspond to *gfap*-positive cells. H : GFAP protein accumulates in bundles of fibres adjacent to rhombomere boundaries. Scale bar : 50μm in G,H, 100μm in A-F.

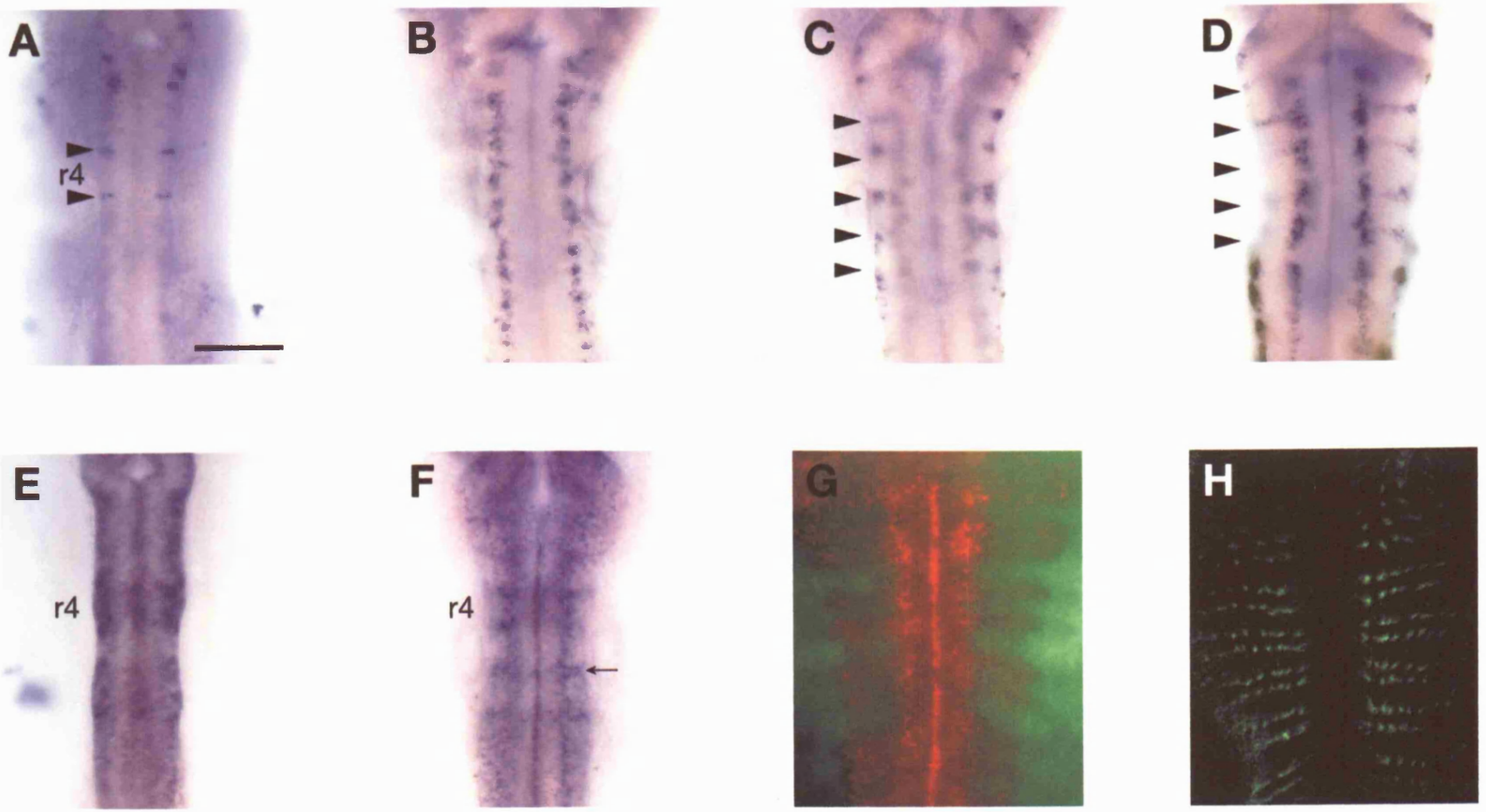
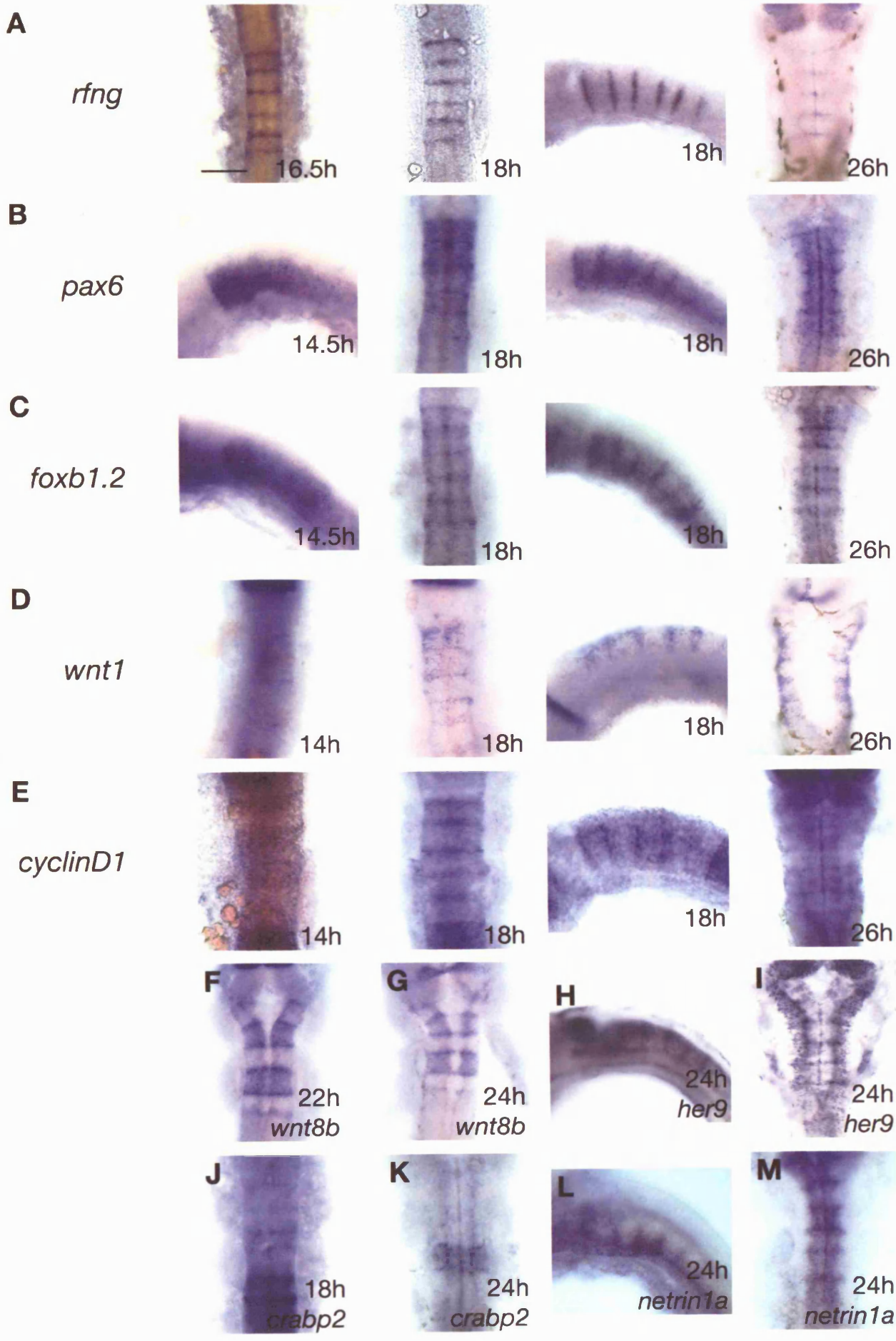

A
r4
B
C
D
E
r4
F
r4
G
H

Fig. 10 : Expression of boundary markers.

Time courses of expression of *rfng* (A), *pax6* (B), *foxb1.2* (C, also called *mariposa*), *wnt1* (D) and *cyclinD1* (E) in rhombomere boundaries. Selected stages of the following markers are also shown : *wnt8b* (F,G), *her9* (H,I), *crabp2* (J,K) and *netrin1a* (L,M). Stages are indicated on the panels. The first and fourth images in A, as well as G and H were obtained from Yi-Chuan Cheng. Scale bar: 100μm.


A
rfng
16.5h
18h
18h
26h
B
pax6
14.5h
18h
18h
26h
C
foxb1.2
14.5h
18h
18h
26h
D
wnt1
14h
18h
18h
26h
E
cyclinD1
14h
18h
18h
26h
F
22h
wnt8b
G
24h
wnt8b
H
24h
her9
I
24h
her9
J
18h
crabp2
K
24h
crabp2
L
24h
netrin1a
M
24h
netrin1a

Differentiation at interrhombomeric boundaries.

Two differentiation markers display a different pattern to the markers described in previous paragraphs, in that they are detected at rhombomere boundaries, suggesting that the cells labelled are derived from boundary cells.

One of these markers is the neuronal gene *evx1* (Thaeron et al., 2000), which is first detected in a few neurons, including ventral reticulospinal neurons in the centre of r2 r5 and r6 (not shown), but can also be seen in dorsal neurons at the r1/r2, r3/r4 and r4/r5 interfaces (see Fig.9A, for 18h). By 24h, two different cell populations are labelled: one more ventral which is adjacent to boundaries (Fig.9B) and more dorsally, clusters of cells at boundaries (Fig.9C). At 26h, a row of evx1-positive cells can be seen aligned at rhombomere boundaries, corresponding to the boundary commissural neurons (Thaeron et al., 2000; Trevarrow et al., 1990), as well as clusters either side of the boundaries (Fig.9D).

The glial marker *gfap* labels both astrocytes and radial glia in many species including zebrafish (Kawai et al., 2001; Levitt and Rakic, 1980; Marcus and Easter, 1995; Nielsen and Jorgensen, 2003). Although the protein distribution has been described at 48h as forming the "glial curtain" either side of boundaries (Trevarrow et al., 1990), the localisation of the cell bodies has not been described. *In situ* hybridisation allows this question to be addressed, as it labels cell bodies. Early expression of *gfap* is segmental (18h, Fig.9E), with low levels present throughout the neural tube, and at higher levels in rhombomeres 4 and 6. By 24h (Fig.9F), expression continues to be restricted to the ventricular zone, and elevated levels can be detected ventrally in boundaries from about 22h. By 30h, the expression domain at boundaries has split into two stripes adjacent to boundaries. Double labelling with the Hu antibody shows that *gfap* and Hu do

not colocalise, indicating that the gaps in Hu staining adjacent to boundaries do indeed correspond to glial cells (36h, Fig.9G). As described previously, fibres detected by an antibody to GFAP are found in bundles projecting ventrally adjacent to boundaries (Fig.9H and Trevarrow et al., 1990). Thus, both the cell bodies and bundles of fibres are present adjacent to boundaries.

Characterisation of gene expression at rhombomere boundaries.

In order to gain insight as to the mechanism of boundary formation, I characterised in detail the expression patterns of various known and novel genes expressed in boundaries. The order of their induction provides clues as to their potential hierarchical relationships, as well as the roles of boundaries. To this effect, I have carried out *in situ* hybridisation with RNAs for boundary genes, in conjunction with Yi-Chuan Cheng, who kindly provided me with two images on Fig.10A, and Fig.10F,G. Confirmation of the localisation of transcripts with respect to the odd/even segment interfaces was obtained by fluorescently co-labelling embryos with EphA4 immunohistochemistry (not shown).

The earliest available boundary marker is *wnt1* (Fig.10D), a member of the Wnt family of secreted signalling molecules. In the hindbrain, *wnt1* is expressed in the roof plate of rhombomeres 2, 3 and 5 at the 10 somite stage (14h). This segmental restriction remains until about 18h ; by 26h, continuous roof plate expression is seen. Boundary expression is first seen from about the 10 somite stage (14h) as stripes of stronger expression compared to the roof plate expression, which at this stage is segmental and restricted to r2, r3 and r5. This expression increases in dorso-ventral extent and at 16.5h and 18h, *wnt1*

transcripts are detected in the dorsal half of rhombomere boundaries. By 26h, boundary expression has become restricted to a more dorsal population. A known downstream target of Wnt1 is the cell cycle regulator *cyclinD1* (Megason and McMahon, 2002; Shtutman et al., 1999; Tetsu and McCormick, 1999), which is expressed in boundaries also from 14h, (Fig.10E). At 18h, high levels of expression are seen throughout hindbrain boundaries, with weaker expression in rhombomere centres. By 26h, in addition to the boundary domains, high expression levels are also detected in stripes in the rhombomere centres. Another member of the Wnt family, *wnt8b* is also expressed in the dorsal half of rhombomere boundaries. At 18h, *wnt8b* expression is restricted to r3 and r5 (not shown). The expression at boundaries is gradually upregulated and is readily detectable from 20h (not shown, see Fig.10F for 22h), while that in the segment centres is downregulated (Fig.10G). *wnt8b* transcripts are also present at the r1/r2 and r6/r7 boundaries, suggesting that boundary expression is not simply due to the downregulation of expression in the centre of r3 and r5.

Boundary expression of *pax6*, a paired box transcription factor, has been described both in chick (Heyman et al., 1995) and in zebrafish (Xu et al., 1995). *pax6* is expressed in the medial part of the hindbrain along the dorso-ventral axis, making it difficult to observe when boundary expression first occurs. The earliest that elevated expression at boundaries can be detected is at the 11 somite stage (14.5h, Fig.10B), as dorsal protrusions from the expression domain, and is readily detected from about the 14 somite stage (16h, not shown). Subsequently, boundary expression is visible as areas of higher intensity of staining. Non-boundary expression is stronger in r2 and r3 at early stages, but by 26h, it is uniform. A similar pattern is observed for *foxb1.2* (Fig.10C, also called *mariposa*

and *fkd3*, Moens et al., 1996; Odenthal and Nusslein-Volhard, 1998), although no indication of boundary-specific expression is detectable at 14.5h. Transcripts can be seen to accumulate at higher levels in boundaries around the 14-15 somite stage (16-16.5h, not shown), and at 18 and 26h looks similar to *pax6*, except that the difference in intensity of staining between boundary and non-boundary is more evident.

The Notch pathway component *radical fringe* (*rfng*) has recently been described (Cheng et al., 2004; Qiu et al., 2004), and is the most specific boundary marker observed to date. Expression is first seen at the 14 somite stage (16h, not shown), and by 16.5h (Fig.10A), all boundaries are labelled. In the hindbrain, no expression can be detected outside rhombomere boundaries. Expression persists until 26h, when it begins to be downregulated, and has disappeared by 27h. Studies by Yi-Chuan Cheng have shown that *rfng* transcripts are detected in two rows of cells straddling the morphological constriction between segments (Cheng et al., 2004).

Other markers that label boundary cells include *her9*, a *hairy*-related transcription factor cloned by a former lab member (Andrea Pasini). *her9* expression is very dynamic in the hindbrain, with higher expression levels in boundaries at 24h, and a punctate pattern in the rhombomere centres (Fig.10H,I). *crabp2* (*cellular retinoic acid binding protein 2*) is also transiently detected in all boundaries at around 18h (Fig.10J), with stronger expression in posterior boundaries, and in r6 and more caudally. It is rapidly lost from more anterior boundaries, and gradually downregulated in the hindbrain, where expression persists only in r6 by 24h (Fig.10K), making this the earliest gene to be downregulated from boundaries. Finally, *netrin1a*, a member of the Netrin

family of growth cone guidance molecules, is also detected in boundaries. *netrin1a* transcripts are present in the ventral half of boundaries from about 18h (not shown, Fig.10L,M), as well as at the ventral midline throughout the hindbrain and spinal cord. Higher levels of expression can also be distinguished in ventral rhombomere 4.

Conclusions.

The data presented here show that spatial patterns of neurogenesis are regulated along the anterior-posterior and dorso-ventral axes within each rhombomere. At early stages, until about 18hpf, neurogenesis occurs ventrally in the centre of rhombomeres, but after this stage, most neurogenesis occurs adjacent to rhombomere boundaries, in a pattern that progresses in a ventral to dorsal manner. Two cell types are found at rhombomere interfaces : *evx1*-positive neurons and *gfap*-expressing radial glial cells.

Moreover, these results show that rhombomere boundaries have dynamic gene expression profiles and that they are patterned along the dorso-ventral axis, with *wnt1* and *wnt8b* dorsally and *netrin1a* ventrally. Different levels of expression of *crabp2* in different boundaries also suggest that boundaries are patterned along the anterior-posterior axis. They also show that the most specific boundary marker is *rfng*, as it is the only gene to be restricted to boundaries at all stages observed, making *rfng* the only clear marker of boundary fate.

The patterns of neurogenesis observed could be explained by a role of boundaries in organising hindbrain neuronal differentiation. In particular, the

secreted factors expressed by boundaries are good candidates for mediating a possible signalling role of boundaries.

Discussion.

Previous work had established that neuronal organisation in the zebrafish hindbrain is patterned along the anterior-posterior axis within each rhombomere (Hanneman et al., 1988; Trevarrow et al., 1990). I have further investigated this organisation using pan-neuronal, differentiation and subtype-specific markers, and I have found that distinct neuronal and glial subtypes are found at distinct anterior-posterior and dorso-ventral locations within each rhombomere. Two possible models can be imagined to explain this organisation : either a morphogen gradient patterns neural subtype specification depending on the location of each cell along the anterior-posterior axis, and/or an affinity or migration guidance gradient underlies the positioning of each cell type after its specification.

Distinct phases of neurogenesis.

The characterisation of neurogenesis in wild type embryos presented here shows that at least three distinct phases of neurogenesis can be distinguished in the zebrafish hindbrain. The first is the development of individually identifiable neurons at stereotypical locations. Although no specific markers are available at early stages for reticulospinal neurons, most of the Hu-positive neurons seen at 10 and 15 somites (Fig.6A,B) are likely to be primary reticulospinal neurons, based on the stereotypy of their location, and the timing of their appearance (Mendelson, 1986; Metcalfe et al., 1986). Indeed, Mendelson was not able to detect any other neurons that became post-mitotic earlier than or at the same time as the first reticulospinal neurons. Unexpectedly, however, some early-born neurons are found at rhombomere interfaces.

The second phase of neurogenesis occurs between 15 somites (16.5h) and 22-24h, when neurons differentiate mainly in the centre of rhombomeres, and few if any additional neurons are seen at interfaces. This pattern has been described previously (Hanneman et al., 1988; Trevarrow et al., 1990) and suggests a block in neurogenesis at boundaries. Recent work in this lab has shown that Notch signalling has a role in preventing premature neuronal differentiation at rhombomere boundaries (Cheng et al., 2004). It is intriguing that neurons are clustered at this stage (see Fig.6C), and there are two potential explanations : either that the differentiating progenitors themselves are clustered, or, as is the case for motor neuron pools in the spinal cord (Price et al., 2002), the post-mitotic neurons assemble into clusters. Here, it appears that patterning

occurs at the level of differentiation itself, as the distribution of *p27^{xic1}-a* in post-mitotic progenitors matches quite closely that of Hu antigens in differentiating neurons. For example in rhombomere 4, differentiation occurs in a broader area, reflected in the lower clustering of Hu-positive cells in r4 at 24h (see Fig.6C and Fig.7G).

Finally, in the third stage, neurogenesis occurs predominantly adjacent to boundaries, where it progresses from ventral to dorsal over time, as revealed by many differentiation markers (*ngn1*, *ashb*, delta genes, *p27^{xic1}-a*, see Fig.7), by markers of individual neuronal subtypes (such as *dbx1a*, see Fig.8) and by several other markers, including some subunits of the neuronal sodium-potassium ATPase, (Canfield et al., 2002) or noradrenergic neuron markers (Holzschuh et al., 2003). Most neurogenesis appears to have taken place by 48h, based on the decreasing expression of proneural genes (see Fig.7P,R and Lyons et al., 2003).

Early neurogenesis produces stereotypically positioned neurons.

Unexpected periodicity and distribution of early neurons.

Previous descriptions of early-born neurons were made either in 5 day-old larvae (Mendelson, 1986; Metcalfe et al., 1986), or at 18h using Nomarski optics or acetylcholinesterase reactivity and immunoreactivity to the zn-1 antibody (Hanneman et al., 1988). These methods are susceptible to lead to an underestimation of the number of neurons present, and are not informative as to

the timing of appearance of these neurons, as the morphology and gene expression markers used may be later events in differentiation. The study by Hanneman *et al.* (1988) had reported that the earliest neurons to differentiate were located in the centre of each segment. By looking at an early pan-neuronal marker, I have made slightly different observations. First, and similarly to what has been described for neurogenesis in the chick hindbrain (Lumsden and Keynes, 1989), the earliest neurons are found in even-numbered rhombomeres (Fig.6A), suggesting a two-segment periodicity. Second, neurons are also found at the r2/r3 and r4/r5 segment interfaces at the 10 somite stage. By the 15 somite stage (16.5h), neurons are found in the centres and at the interfaces of most segments (with the apparent exception of the r5/r6 interface), indicating a half-segment periodicity, which has been briefly alluded to by Hanneman *et al.* (1988), but only for the two known reticulospinal neurons at the borders of r4, the MiR class. However, the presence of neurons at the r1/r2 and r2/r3 interfaces, the latter being among the earliest neurons, cannot be accounted for if they are reticulospinal neurons. This implies either that a small number of neurons of a different kind differentiate as early as the earliest reticulospinal neurons, or that certain reticulospinal neurons migrate from their place of origin at rhombomere boundaries towards the centre of each rhombomere.

How is early neurogenesis patterned ?

An important question arises as to what determines the location of each particular neuron, given the stereotypical nature of its position. The difficulties of this question are particularly evident in the case of the Mauthner cell, which is positioned in the centre of r4 and is born around 7.5h of development, during gastrulation (Mendelson, 1986). While it has been shown that specification of the Mauthner cell requires at least one of the two zebrafish *hoxb1* genes, it was found that a knockdown of *hoxb1b*, which is expressed between 6-10h in r4, is not sufficient to cause a loss of this cell, while the second gene, *hoxb1a*, is not expressed until 10h (McClintock et al., 2002). The latter authors argue that this reflects that the identity of the Mauthner cell is not determined for some time after it leaves the cell cycle. Similarly, several other reticulospinal neurons are born around 9.5h, among which some are found in r5 and r6 (Mendelson, 1986). This is roughly 30 minutes before the segmentation genes *krox20* and *valentino* are expressed (Moens et al., 1998; Oxtoby and Jowett, 1993). Although this timing difference may not be significant, the patterning of segments at these stages is still coarse (see Cooke and Moens, 2002), which makes it difficult to comprehend how precise patterning of the positioning of these neurons is eventually attained.

There are several potential explanations for how this pattern is obtained. One explanation could be that segmentation occurs earlier than is thought based on current markers. Nevertheless, the early expression patterns of segmental markers are coarse (Cooke and Moens, 2002; Irving et al., 1996b), implying that even if this is the case, an early pre-pattern cannot account for later precise positioning of individual cells. Therefore, it seems more likely that a mechanism

of progressive refinement of pattern operates. Taking for instance the case of the Mauthner neuron, it is conceivable that it is specified as a neuron within a broad domain encompassing rhombomere 4 when only coarse anterior-posterior patterning has been established, and that the later expression of Hox genes confers its specific identity to this neuron. Precise positioning of the Mauthner must require cell movement, as at early stages each rhombomere is approximately 2-3 cells wide.

The most likely explanation for the stereotypical position of each early-born neuron is that of an affinity gradient within each rhombomere. This idea has been proposed to play an important role in reducing intrinsic cell mobility and therefore maintaining sharp patterning in segments in the *Drosophila* ectoderm (Lawrence et al., 1999). In the hindbrain, such a gradient could similarly stabilise pattern and ensure that each cell is located in a precise location. Unlike neuronal specification, however, establishment of the cues that regulate affinity within each rhombomere requires that segmentation is fully completed, as the source of these cues must be precisely located, in the same way that morphogen sources must be precisely regulated (Dahmann and Basler, 1999). Evidence for this is provided by the *hoxb1* knockdowns alluded to above (McClintock et al., 2002), in which the Mauthner neuron, when still present in double *hoxb1* and *hoxb1b* knockdowns, is often shifted relative to rhombomere interfaces.

Late neurogenesis is segmentally reiterated.

The second and third phases of neurogenesis both occur in patterns which are repeated from segment to segment, first in the centre of each rhombomere, then adjacent to boundaries. There are differences between the neurons produced in different segments, particularly in the case of cranial motor neurons, but overall, the general pattern of neuronal production in each segment suggests more similarities than differences, and that a similar mechanism of anterior-posterior patterning occurs in all segments. The ventral and dorsal midline structures (floor and roof plates) are known regulators of patterning and differentiation along the dorso-ventral axis (Briscoe and Ericson, 2001; Lee and Jessell, 1999; Megason and McMahon, 2002). By analogy, it is likely that signalling centres pattern the anterior-posterior axis within segments, and rhombomere boundaries are the best candidates. However, it is interesting to note that it is not the identity of cells that is regulated along the anterior-posterior axis, but the location of their differentiation. For instance, the ventricular expression of *dbx1a* is continuous along the anterior-posterior axis within segments, while stripes of differentiated neurons are present only adjacent to boundaries. This implies that the post-mitotic neurons have stayed at the anterior-posterior location where they differentiated. Considering that other types of neuron do migrate along the anterior-posterior axis after differentiation (subsets of cranial motor neurons, for example, Chandrasekhar et al., 1997), it can be postulated that there is a mechanism to stabilise the localisation of differentiating neurons along the anterior-posterior axis after they leave the ventricular zone. This mechanism could consist either of an affinity gradient along the anterior-posterior axis within

each rhombomere, with the properties of each neural cell type assigning its location along this gradient, or local migration cues to which each cell type responds independently. The former model is most likely, due to its simplicity, but it is not excluded that there are certain types of neuron that respond to local guidance cues during active migration, as has been demonstrated for the facial motor neurons (Schwarz et al., 2004).

Thus, the regulation of neural patterning in the hindbrain involves an unexpected mechanism. The characteristic anterior-posterior pattern within each segment (Trevarrow et al., 1990 and this work) may be obtained not by a morphogen gradient, but by a combination of spatially-restricted differentiation of progenitors and affinity- or migration-driven localisation of post-mitotic cells. The most likely source of a potential signal regulating both affinity and the location of differentiation are the rhombomere boundaries. Indeed, they express at least one secreted signalling molecule, *wnt1*. In the chick spinal cord, Wnt1 has been shown to regulate the spatial distribution of differentiation, by promoting proliferation of progenitors and inhibiting neuronal differentiation, thus providing an explanation for the observed ventral to dorsal order of differentiation (Megason and McMahon, 2002). This raises interesting questions about the role of Wnt1 in the zebrafish hindbrain, as at 18h neuronal differentiation occurs ventrally in the centre of rhombomeres, distant to the sources of Wnt1 in the roof plate and hindbrain boundaries. However, the fact that, at 24h and later stages, neurogenesis occurs adjacent to boundaries suggests that at these stages, boundaries may positively regulate neuronal differentiation.

Comparison between late differentiation patterns and accumulation of Hu-labelled cells.

Early studies of neurogenesis in the hindbrain did not reveal the pattern of differentiation in stripes adjacent to boundaries. This is most likely due to the limitation in the availability of markers at the time. However, this poses an important question of why this pattern cannot be seen, for instance using markers which label all neurons (such as Hu immunohistochemistry). Indeed, considering that most differentiation takes place adjacent to boundaries, and that at least some neuronal subtypes, such as *dbx1a*-expressing neurons, remain in this position, one would expect to see more neurons adjacent to boundaries than in the centre of rhombomeres, particularly in dorsal regions where there is no differentiation at early stages, yet no such organisation can be seen. Post-mitotic neurons seem equally distributed throughout the non-boundary regions. It remains possible that the discrepancy between the two patterns is due to a lack of available markers and that other genes drive differentiation in centre regions. For instance, there are at least two homologues of the *Xenopus* $p27^{xic1}$ and one gene closely related to the mammalian $p27^{kip1}$ in zebrafish (Geling et al., 2003). However, this idea is inconsistent with the expression pattern of Delta genes. Therefore, in order to account for the discrepancy between neurogenesis patterns and the arrangement of post-mitotic neurons, it seems likely that some cells migrate away from their point of birth adjacent to boundaries towards rhombomere centres. To determine whether this is the case, it would be necessary to have a set of markers labelling various identified subsets of neurons and observe their positions along the anterior-posterior axis. However studies are limited by the fact that the dorso-ventral patterning of the neural tube has been

less studied in the zebrafish than in other species, and therefore that it is harder to assign a transcription factor code to individual cell types, as has been done for chick and mouse (Briscoe et al., 2000; Lee and Jessell, 1999). Nevertheless, individual markers can help to give information about neuronal types. For instance, *gata3* is initially expressed at 24h in small clusters of ventral neurons either side of rhombomere boundaries (data not shown, see also the Zebrafish Information Network expression data at www.zfin.org, referenced in Sprague et al., 2003), but by 30h, a single large cluster of expressing neurons is found in what appears to be the centre of each rhombomere (Neave et al., 1995). This could indicate that this particular group of neurons is born adjacent to boundaries but migrates to the centre of each rhombomere, but could also be due to different cells expressing this same gene at different times in development. However, the evidence that some differentiating neurons migrate to the centre of rhombomeres consolidates the idea of a two-step model for neural patterning : first differentiation is localised to areas adjacent to boundaries, then individual neurons respond to local or graded cues to find their final location.

Differentiation at rhombomeric interfaces.

Neurogenesis at boundaries.

As described in previous work, differentiation is delayed at boundaries in a Notch-dependent manner (Cheng et al., 2004). Interestingly, it is when the Notch pathway component *rfng* is downregulated in boundary cells that significant numbers of boundary neurons start to be detected (around 27h).

However, in the *mind bomb* mutant, where most, but not all, Notch function is removed (Cheng et al., 2004; Itoh et al., 2003), there are still gaps in neurogenesis at some boundaries. Delta gene expression is defective in *mind bomb* embryos (Bingham et al., 2003), such that boundary cells experience less activation of Notch. However, it is likely that residual Notch activation occurs in *mind bomb* which is sufficient to partially inhibit neurogenesis.

So far, only one type of neuron has been identified at rhombomere boundaries, which is a population of *evx1*-positive commissural neurons (Thaeron et al., 2000). It is probable that there are also other types of neurons located at boundaries, as *evx1*-positive cells only occupy a particular dorso-ventral location at the boundary, while Hu-positive neurons are found throughout the dorso-ventral axis of boundaries. The origin of neurons found at interfaces remains to be established, as no markers of neurogenesis can be seen at boundaries at any stage examined. It is therefore possible that boundaries are non-neurogenic and that the *evx1*-positive neurons are derived from progenitors located in the non-boundary regions. Alternatively, after asymmetric division, the daughter of a boundary cell could find itself in the progenitor domain adjacent to boundaries, and thus give rise to neurons. Another possibility is suggested by the observation that boundary neurons and *gfap*-expressing cells start to be seen around the same stage. It has been shown recently that the radial glia that form the glial curtain are proliferating (Lyons et al., 2003), and this suggests interesting analogies with studies in the mouse cortex, where radial glia have been shown to be an important population of neuronal progenitors (Gotz et al., 2002; Malatesta et al., 2003; Noctor et al., 2002).

Origin and role of radial glia adjacent to boundaries.

Although no lineage analyses have been carried out, several lines of evidence suggest that the radial glial cells that differentiate adjacent to hindbrain boundaries may be boundary-derived. First, *in situ* hybridisation with *gfap* labels cells in single stripes located at boundaries at 22-24h (Fig.9), although these stripes later split in two. Moreover, when boundary markers expand in ectopic situations, such as in *wnt1* knockdowns, *gfap* expression also expands (Fig.18O-R). This is consistent both with the lack of expression of proneural genes in these morphants, as well as with expansion of boundary fate, but it is difficult to distinguish these two explanations. Indeed it may be significant that boundary fate and glial/non-proneural fate appear to be linked, as boundary cells do not express proneural genes.

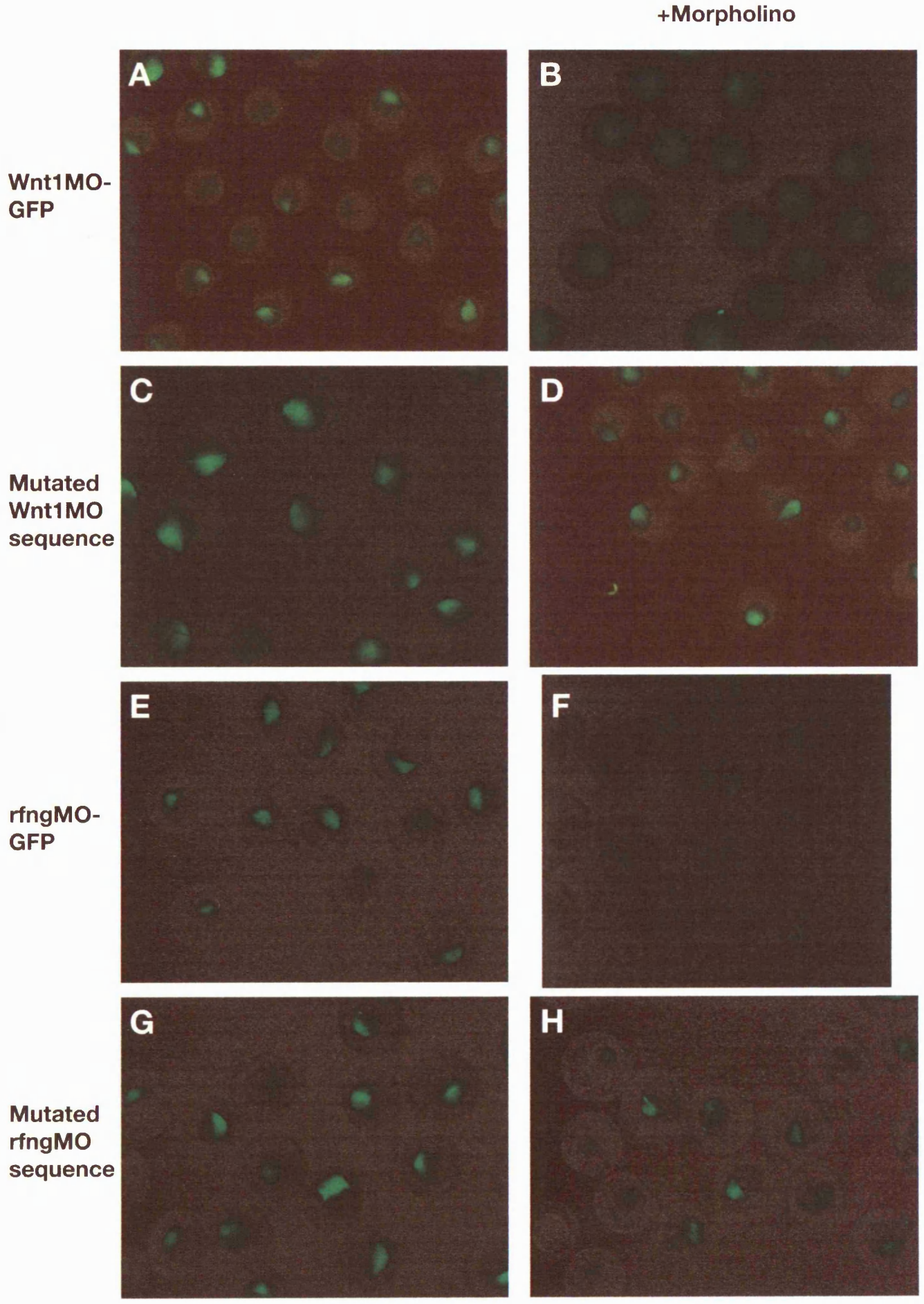
The role of the radial glia adjacent to boundaries is unclear. One possibility is that they guide commissural axons (Trevarrow et al., 1990). It is also conceivable that they are neuronal progenitors for non-boundary neurons, like radial glia in the mammalian cortex which are neuronal progenitors (Gotz et al., 2002; Malatesta et al., 2003; Noctor et al., 2002). Although this could account for the stripes of neurogenesis adjacent to boundaries, it is unlikely as stripes of proneural genes can be seen before the appearance of clear *gfap*-expressing stripes. It is possible that these radial glial cells are part of a pool of progenitors required for future neurogenesis (Lyons et al., 2003). Finally, it is also likely that the cell bodies of neurons differentiating adjacent to hindbrain boundaries migrate along the radial glial fibres to the mantle zone, as occurs in the mammalian cortex (Rakic, 1978).

The role of Wnt signalling in patterning hindbrain boundaries and neurogenesis.

The distribution of neuronal and glial cell types that I have characterised above suggests that boundaries may have a role in patterning the anterior-posterior axis of each rhombomere in the zebrafish hindbrain. This is the hypothesis I have set out to test in the second part of my thesis, where I have knocked down genes expressed in boundaries. The best candidate for carrying out a potential patterning role is Wnt1, a secreted extracellular signalling molecule, which is expressed by boundary cells (see Fig.10D). In my analysis I tested first the effect of *wnt1* knockdown on the expression of boundary genes, in order to establish a hierarchy among genes expressed in boundaries, and second, whether perturbing boundary genes has an effect on expression patterns in non-boundary regions. At the same time, Yi-Chuan Cheng, a postdoc in the Wilkinson lab, analysed the role of the Notch pathway, given the striking expression pattern of *rfng* and the exclusion of *delta* genes from boundaries. As will be discussed, we found that these genes are linked in a regulatory pathway.

Fig. 11 : Specificity and efficacy of morpholino oligonucleotides.

Low-magnification images of sphere stage (4h) live embryos injected with RNA constructs encoding nuclear GFP containing the morpholino oligonucleotide recognition sequences for wnt1MO (A,B), mutated wnt1MO (C,D), rfngMO (E,F) or mutated rfngMO (G,H). Co-injection of the wnt1MO (B,D) or rfngMO (E,H) blocks GFP expression (B,F), but not from the RNAs in which the sequence has been mutated (D,H).


+Morpholino
A
B
Wnt1MO-GFP
C
D
Mutated Wnt1MO sequence
E
F
rfngMO-GFP
G
H
Mutated rfngMO sequence

Results.

Specificity and efficacy of morpholino oligonucleotides.

The role of *wnt1* was assessed by using a morpholino oligonucleotide (MO) to block *wnt1* translation. In order to test that the morpholinos I have used efficiently block translation, I assayed their effectiveness in *in vivo* conditions. I inserted the nucleotide sequence that is recognised by either wnt1MO or rfngMO (morpholino against *rfng*, see below), at the start site of a nuclear localised green fluorescent protein (GFP) cDNA. For each of the morpholinos tested, I also made a construct differing from the morpholino recognition sequence by five random nucleotides. RNA was then synthesised from these recombined GFP cDNAs and injected into one to four cell stage embryos. Injected alone, each of the RNAs gives robust GFP expression (Fig.11A,C,E,G). Injection of wnt1MO blocks GFP fluorescence when co-injected with the RNA containing the wnt1MO recognition sequence (Fig.11B), but not with the RNA containing the mutated sequence (Fig.11D). Similarly, rfngMO can block GFP expression from the RNA containing the rfngMO recognition sequence (Fig.11F), but not the mutated sequence (Fig.11H). These results suggest that the morpholinos tested can efficiently and specifically inhibit translation from RNAs containing their recognition sequence. In the case of rfngMO, all the results presented below were confirmed using a second morpholino oligonucleotide with a recognition sequence that lies upstream of the start codon (see Cheng et al., 2004). All the images shown in this thesis have been obtained from experiments with the ATG-overlapping morpholino. Finally, in the case of the morpholino against *tcf3b*, the

same sequence was used as previously published (Dorsky et al., 2003), where it was shown that this morpholino can block translation of the *tcf3b* gene product *in vitro*.

Gross morphological effects of* wnt1 *knockdown.

Embryos injected with wnt1MO are slightly smaller than their wild type siblings, and the neural tube fails to open in the hindbrain, and is thinner. Nevertheless there are no other obvious defects in the embryos up to 48h ; the midbrain-hindbrain region appears grossly normal morphologically, unlike what has been described in the mouse for a *wnt1* knockout (McMahon and Bradley, 1990; McMahon et al., 1992).

Knockdowns of the Wnt pathway cause expansion of boundary markers.

In the first set of experiments, I tested the possibility that Wnt1 might have a role in the formation or organisation of hindbrain boundaries.

Timing and effect of* wnt1 *knockdown on boundary marker expression.

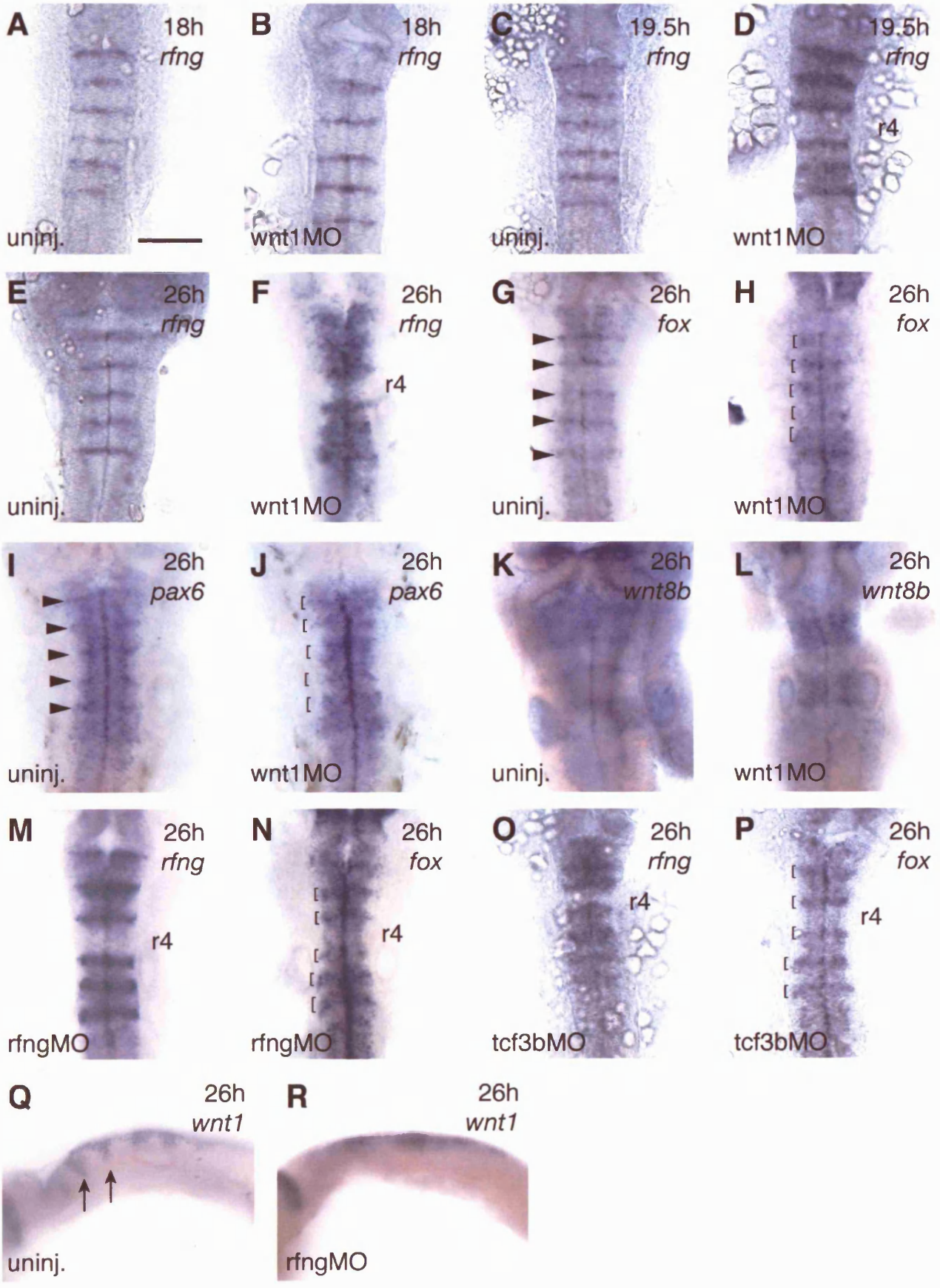
I detected the expression of *rfng* and *foxb1.2* boundary markers in *wnt1* MO-injected (wnt1MO) embryos. These genes are independent markers of hindbrain boundaries, since knockdown of either does not lead to decreased expression of the other (see below, and data not shown). At 18h, no effect on

rfng expression can be observed (Fig.12A,B). In contrast, only 1.5 hours later, at 19.5h (21 somite stage), *rfng* expression is much broader and extends into the centre of rhombomeres, except rhombomere 4 (Fig.12C,D). By 24h, expression of *rfng* can be seen in most of the hindbrain, with the exception of rhombomere 4 (Fig.12E,F). Similarly, the boundary domain of *foxb1.2* expression is expanded in wnt1MO embryos (Fig.12G,H, brackets indicate broadened boundary stripes), although, due to the non-boundary expression domain of *foxb1.2* in the ventricular zone, this is less evident than for *rfng*.

It is more difficult to discern whether there is a similar effect on *pax6* expression (Fig.12I,J), since the boundary and non-boundary expression levels are hard to distinguish. However, broadened stripes of expression are discernible in injected embryos (brackets in Fig.12J), consistent with an expansion of the boundary domain of expression. In control embryos at 24h, *wnt8b* is expressed in rhombomere boundaries, and more weakly in dorsal r3 and r5 (12K). In wnt1MO-injected embryos, *wnt8b* is not upregulated in boundaries, and is expressed at high levels in r3 and r5. This expression is reminiscent of the earlier expression pattern of *wnt8b*, up to 18h, when transcripts are only detected in dorsal r3 and r5.

Fig. 12 : Knockdowns of the Wnt pathway cause expansion of boundary markers.

Dorsal views of boundary marker expression in uninjected, wnt1MO-injected (B,D,F,H,J,L), rfngMO-injected (M,N) and tcf3bMO-injected (O,P) embryos. Stage and markers are indicated in the top left hand corner of each panel. A-F : Time course of the expansion of *rfng* expression in wnt1MO embryos showing that between 18h and 19.5h, *rfng* expression spreads to fill most of the hindbrain, except rhombomere 4 (r4). G-L : expression of *foxb1.2*, *pax6*, and *wnt8b* showing the effect of wnt1MO injection. Arrowheads in G and I point to sharp stripes of boundary expression in uninjected embryos, as compared to the broader domains in all three morphants (brackets in H,J,N,P). M-P : expression of *rfng* and *foxb1.2* in rfngMO and tcf3bMO embryos, displaying a similar phenotype to wnt1MO embryos. Q,R : boundary expression of *wnt1* (arrows in Q) is lost in rfngMO embryos (Q and R from Yi-Chuan Cheng, see Cheng *et al.*, 2004). Scale bar : 100μm.


A
18h
rfng
uninj.
B
18h
rfng
wnt1MO
C
19.5h
rfng
uninj.
D
19.5h
rfng
r4
wnt1MO
E
26h
rfng
uninj.
F
26h
rfng
r4
wnt1MO
G
26h
fox
uninj.
H
26h
fox
wnt1MO
I
26h
pax6
uninj.
J
26h
pax6
wnt1MO
K
26h
wnt8b
uninj.
L
26h
wnt8b
wnt1MO
M
26h
rfng
r4
rfngMO
N
26h
fox
r4
rfngMO
O
26h
rfng
r4
tcf3bMO
P
26h
fox
r4
tcf3bMO
Q
26h
wnt1
uninj.
R
26h
wnt1
rfngMO

Taken together, these findings suggest that Wnt1 is required for the appearance of later boundary markers such as *wnt8b*, as well as to block *rfng* and *foxb1.2* boundary marker expression in the centre of rhombomeres.

rfng *knockdown suggests that the boundary domain of* **wnt1** *expression is required for the repression of boundary markers.*

Recently published work by this laboratory has suggested that boundary cells have elevated Notch signalling and that modulation of Notch activity by Rfng is required for *wnt1* expression in boundaries (Fig12Q,R and Cheng et al., 2004). Moreover, in *rfng* knockdowns, roof plate expression of *wnt1* is maintained, thus allowing me to assay specifically for roles of the boundary domain of *wnt1* expression.

In embryos injected with rfngMO, expression of *rfng* expands to fill entire rhombomeres, with the exception of r4 (Fig.12M), and the boundary expression of *foxb1.2* is also much broader (Fig.12N), the same phenotype as wnt1MO embryos (compare with Fig.12F,H). This result implies that the boundary expression of *wnt1* is required to prevent boundary markers from being expressed ectopically.

The effect of **wnt1** *is mediated by* **tcf3b** *in the hindbrain.*

A candidate mediator of Wnt signal transduction in the hindbrain is the transcription factor *tcf3b*, as *tcf3b* knockdown was previously shown to have a phenotype in the hindbrain which includes a lack of morphological boundaries, and uniform levels of *foxb1.2* expression. The authors interpreted uniform *foxb1.2* expression as absence of boundaries (Dorsky et al., 2003). However, the

non-boundary domain of *foxb1.2* expression makes it difficult to distinguish between an absence of boundaries and an expansion of boundary expression.

To resolve this question, I used the same morpholino as these authors, directed against the *tcf3b* translation start site, and analysed the expression of boundary markers. In tcf3bMO embryos, both *rfng* and *foxb1.2* boundary expression domains expand to fill most of the rhombomere centres (Fig.12O,P), as described above for wnt1MO and rfngMO. The image shown in Fig.12P corresponds to half the dose of tcf3bMO usually injected, to show that the *foxb1.2* expression observed does correspond to the boundary domain, as higher doses give a strong phenotype with almost uniform expression of boundary markers, making it difficult to distinguish between the two hypotheses presented above.

The finding that morpholinos against *wnt1* and *tcf3b* give the same phenotype suggests that both morpholinos are specific, and also that these two genes act in the same pathway, that is to say that the effect of Wnt1 in the zebrafish hindbrain is mediated by Tcf3b.

Range of phenotypes in injected embryos.

As is commonly observed in morpholino injections, there is some variability in the severity of the effect observed, perhaps reflecting variations in the amount injected and/or response of the embryo. The embryos shown in the figures are representative of the majority of embryos. However, an accurate representation of the effect of morpholino injections requires that the range of phenotypes observed be presented.

In the case of *rfng* expression in wnt1MO embryos, four distinct phenotypes can be observed. First, in about 7% of embryos (3/41 embryos, these figures correspond to two separate experiments), *rfng* expression is indistinguishable from uninjected or control morpholino-injected embryos (Fig.13A). In the second category, a mild phenotype of boundary expansion is observed, where the stripes of expression are broader, but do not fill the rhombomere centres (Fig.13B). This accounts for about 10% of wnt1MO embryos (4/41 embryos). The third, and most common, phenotype (about 66% of embryos – 27/41) is shown in Fig.13C and Fig.12F. In this instance, *rfng* expression fills most of the hindbrain, except r4 where little expression can be seen. Higher levels of expression are distinguishable around the rhombomere interfaces. In the fourth category, *rfng* expression is very strongly upregulated throughout the hindbrain, and the levels at rhombomere interfaces and centre regions are similar. Expression can be detected in r4, but it is weaker than in other rhombomeres. Approximately 17% of embryos (7/41) correspond to this phenotype.

Only two phenotypes can be distinguished for *foxb1.2* expression after wnt1MO injection, as no embryos were found to resemble uninjected or control morpholino-injected embryos. In the first case, two levels of expression of *foxb1.2* are distinguishable, with the high level boundary expression in broader stripes compared to uninjected embryos (Fig.12H and Fig.13F). This corresponds to 61% of wnt1MO embryos (17/28 from two separate experiments). In the second category, *foxb1.2* expression appears uniform throughout most of the hindbrain, except rhombomere 4, where lower levels are often detected. This accounts for 39% of wnt1MO embryos (Fig.13G).

The effect of *tcf3b* knockdown appears consistently stronger than for *wnt1*, especially in the case of *foxb1.2* expression. When the same dose of morpholino is injected as for wnt1MO, about 75% of embryos show the same phenotype as in Fig.13G, compared to 39% for wnt1MO-injected embryos. However, when half the amount of tcf3bMO is injected, the proportions of each phenotype are comparable to wnt1MO injections. This may account for the differing interpretations of the phenotypes proposed here and in Dorky *et al.* (2003). Finally, in the case of the *rfng* morpholino, the phenotype appears weaker than wnt1MO. In one representative experiment (similar data was obtained by Yi-Chuan Cheng), most embryos show the mild boundary expansion phenotype presented in Fig.13B (72% - 13/18 embryos), and only 6% (1/18 embryos) have boundary marker expression in rhombomere centres, corresponding to the most common wnt1MO phenotype.

In summary, the results shown are consistent for each morpholino, but there are differences in the strength of the phenotype observed with morpholinos targeted to different mRNAs, with tcf3bMO being the most severe, and rfngMO the mildest. Possible explanations for this will be presented in the Discussion.

Fig. 13 : Range of phenotypes for boundary markers.

Phenotypes of *rfng* (A-D) and *foxb1.2* (E-G) following wnt1MO injection. In 7% of embryos (3/41), *rfng* expression is indistinguishable from uninjected (A). 10% (4/41) have broader expression that does not fill entire rhombomeres (B), while 66% (27/41) have expression throughout most of the hindbrain, except in r4, where little ectopic expression is seen (C). In 17% of cases (7/41), ectopic expression in rhombomere centres is indistinguishable from boundary expression (D). For *foxb1.2*, no injected embryos have a similar phenotype to uninjected embryos (E). 61% (17/28) have distinguishable broader boundary expression domains (F), and 39% (11/28) have uniform expression (G). Scale bar : 100μm.

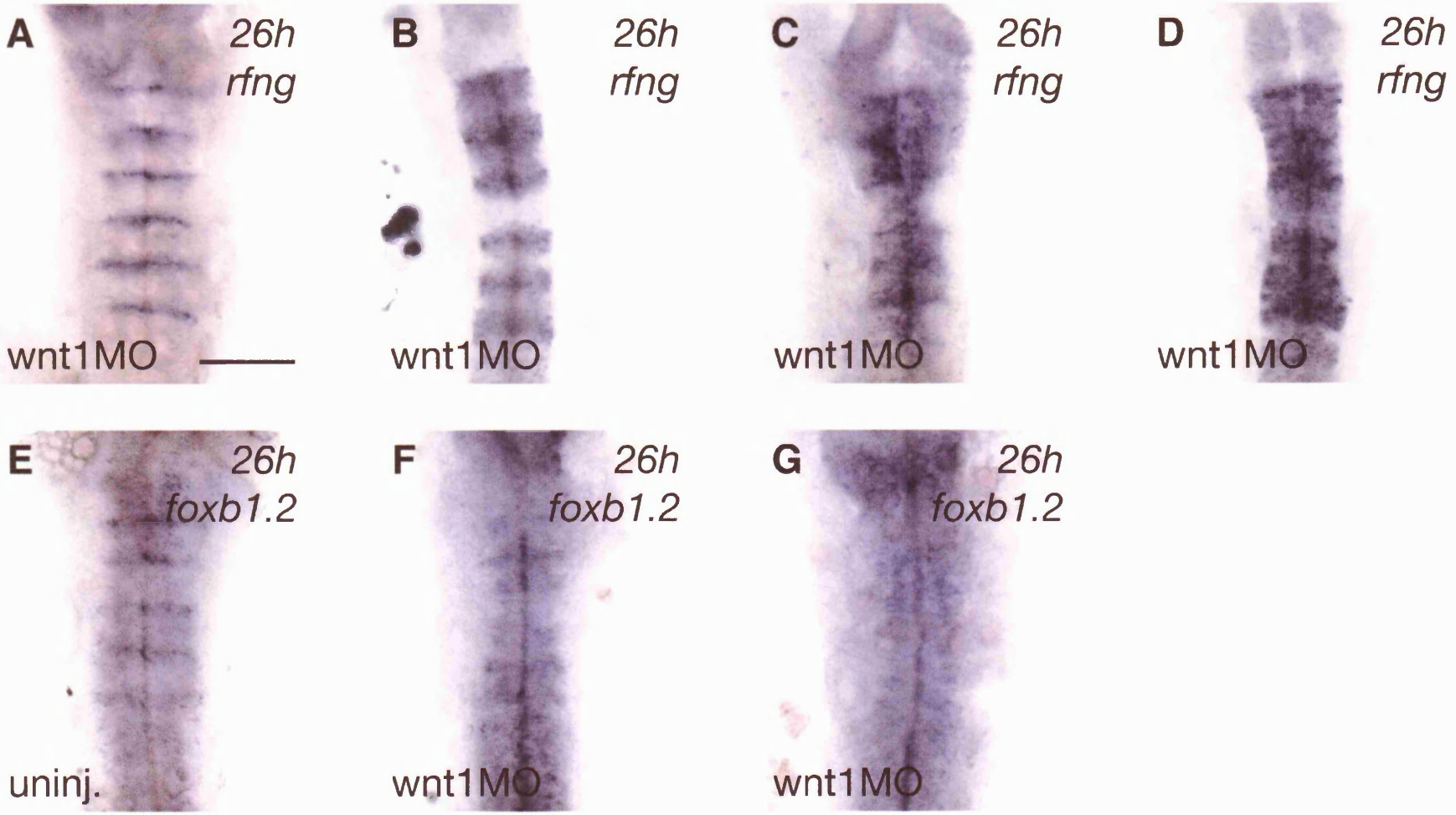
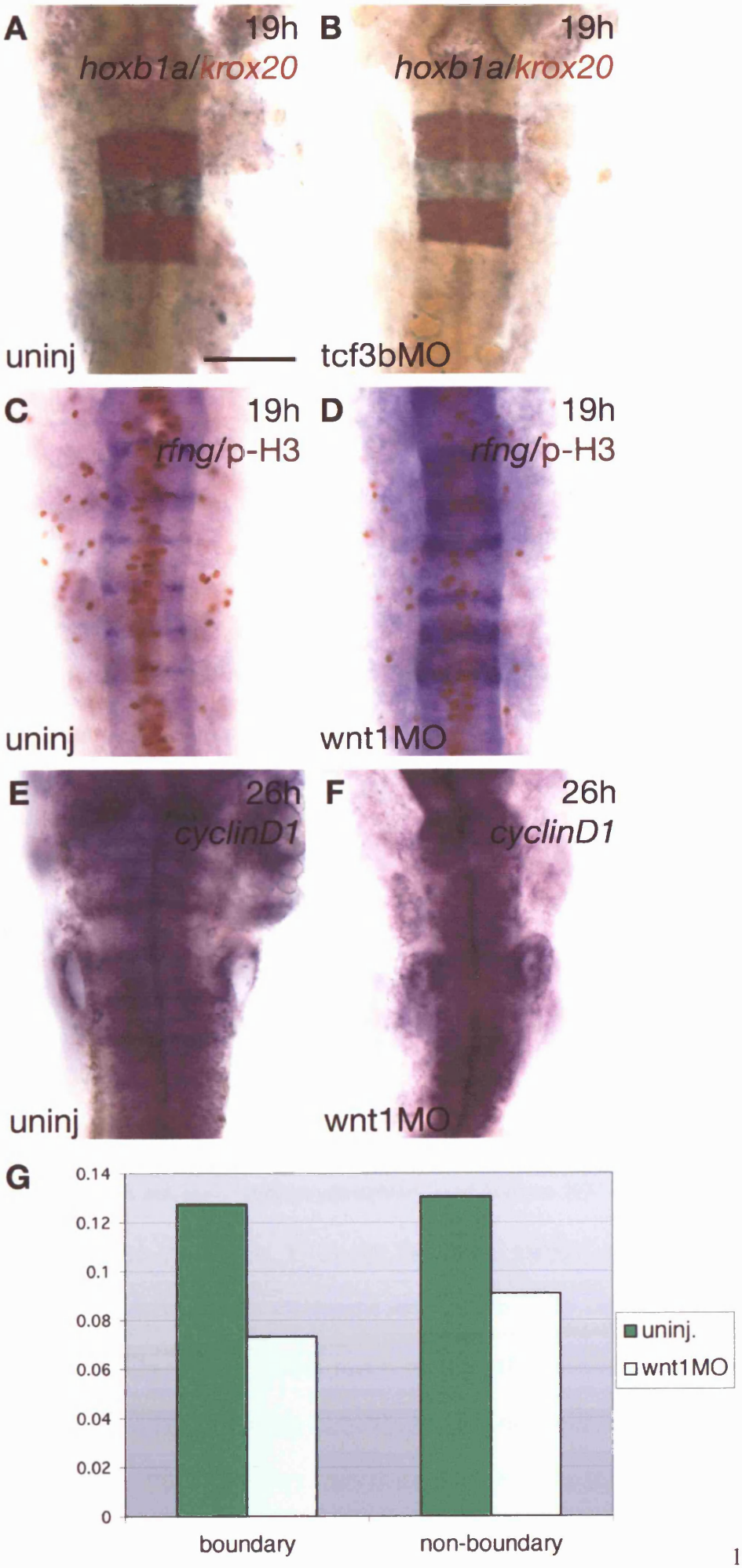

A
26h
rfng
wnt1MO
B
26h
rfng
wnt1MO
C
26h
rfng
wnt1MO
D
26h
rfng
wnt1MO
E
26h
foxb1.2
uninj.
F
26h
foxb1.2
wnt1MO
G
26h
foxb1.2
wnt1MO

Fig. 14 : Mixing between segments and proliferation cannot account for boundary marker expansion in Wnt-pathway morphants.

A,B : *hoxb1a/krox20* double *in situ* hybridisation confirming that the segmentation of the hindbrain is normal in *tcf3b* knockdowns, and that no mixing occurs between segments. C,D : combined *rfng in situ* hybridisation with immunohistochemistry against phosphorylated histone H3 (p-H3, brown) in uninjected and wnt1MO embryos at 19h, from which mitotic ratios, shown in G, were derived. The mitotic ratios of both boundary and non-boundary cells decreases in wnt1MO-injected embryos (42% decrease for *rfng*-positive cells versus 30% decrease for *rfng*-negative cells). *cyclinD1* expression (E,F) also shows that boundary expression is specifically lost in wnt1MO embryos, which suggests that Wnt1 enhances the proliferation of boundary cells, rather than act to restrict it. Scale bar : 100μm.


A
19h
hoxb1a/krox20
uninj
B
19h
hoxb1a/krox20
tcf3bMO
C
19h
rfng/p-H3
uninj
D
19h
rfng/p-H3
wnt1MO
E
26h
cyclinD1
uninj
F
26h
cyclinD1
wnt1MO
G
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
boundary
non-boundary
uninj.
wnt1MO

The expansion of boundary markers is due to* de novo *induction of boundary markers.

Three main hypotheses can be put forward for the observed expansion of boundary markers when signalling through the Wnt pathway is inhibited. The first is that it is due to an increased mixing of cells, either of boundary cells that move away from interfaces, or to an interleaving of interfaces, as boundary markers are upregulated by interactions between cells from neighbouring rhombomeres (Guthrie and Lumsden, 1991). However, there are many more cells expressing boundary markers in Wnt pathway knockdowns, precluding the possibility of a simple migration of boundary cells into rhombomere centres; and the sharp segmental interfaces between the expression domains of *krox20* and *hoxb1* form normally in wnt1MO and tcf3bMO embryos (Fig.14A,B for tcf3bMO, not shown for wnt1MO). Similarly, the limits of *epha4* expression domains are sharp (see for instance Fig.16E).

A second alternative is that the excess number of cells expressing boundary markers reflects a large increase in proliferation of boundary cells and concomitant loss of non-boundary cell proliferation. This seems unlikely, however, as only 1.5 hours are sufficient for most cells in the hindbrain to express *rfng*. Nevertheless, the mitotic index of *rfng*-positive and negative cells was assayed using an antibody against phosphorylated histone H3, which labels mitotic cells. During the stages when the boundary marker expansion is occurring, at 19h of development, the mitotic index of boundary cells decreases by approximately 42%, whereas that of non-boundary cells decreases by about 30% (Fig.14G). Another argument against changes in proliferation being responsible for the ectopic boundary marker expression is the localisation of

cyclind1 transcripts in uninjected and wnt1MO embryos. In the spinal cord, CyclinD1 is a target of Wnt signalling and is thought to mediate the mitogenic effect of dorsal midline Wnts (Megason and McMahon, 2002). However, in the hindbrain, *cyclind1* is expressed at high levels in boundary cells, and lower levels throughout most of the hindbrain. The high boundary levels of *cyclinD1* are specifically lost in wnt1MO embryos (Fig.14E,F), suggesting that Wnt1 acts to enable proliferation in boundaries.

The final and favoured hypothesis, therefore, is that Wnt1 signalling via Tcf3b is required to inhibit non-boundary cells from expressing boundary markers.

Role of the Wnt pathway in regulating hindbrain neurogenesis.

A potential mechanism by which Wnt1 may act on neighbouring cells is suggested by the expression patterns of proneural and delta genes, which, as described previously, are found in stripes of high expression adjacent to boundaries by 24h (Fig.7). This situation is reminiscent of patterning events at the dorso-ventral boundary of the *Drosophila* wing imaginal disc, where Notch signalling upregulates *wingless* at the boundary. In turn, Wingless signals to adjacent cells in the anterior compartment to upregulate *achaete*, *delta* and *serrate*, and specify these as sensory hair cells. Delta cell autonomously blocks Notch activation and therefore prevents these cells from expressing *wingless*, thereby mediating a pathway of lateral inhibition of boundary cells (de Celis and

Bray, 1997; de Celis and Bray, 2000; Diaz-Benjumea and Cohen, 1995; Micchelli et al., 1997; Rulifson and Blair, 1995; Rulifson et al., 1996).

It is intriguing that the homologous genes in the zebrafish hindbrain are expressed with similar spatial relationships as in the *Drosophila* wing imaginal disc. Furthermore, it is known that Notch signalling in hindbrain boundaries activates *wnt1* expression in boundaries (Fig.12Q,R and Cheng et al., 2004). I therefore tested first whether the regulatory relationships between Wnt signalling and *achaete-scute* and *delta* homologues are comparable to those in the wing disc, and second whether proneural genes and *delta* homologues mediate lateral inhibition of boundary fate.

***Regulation of proneural and delta genes by* wnt1 *via* tcf3b.**

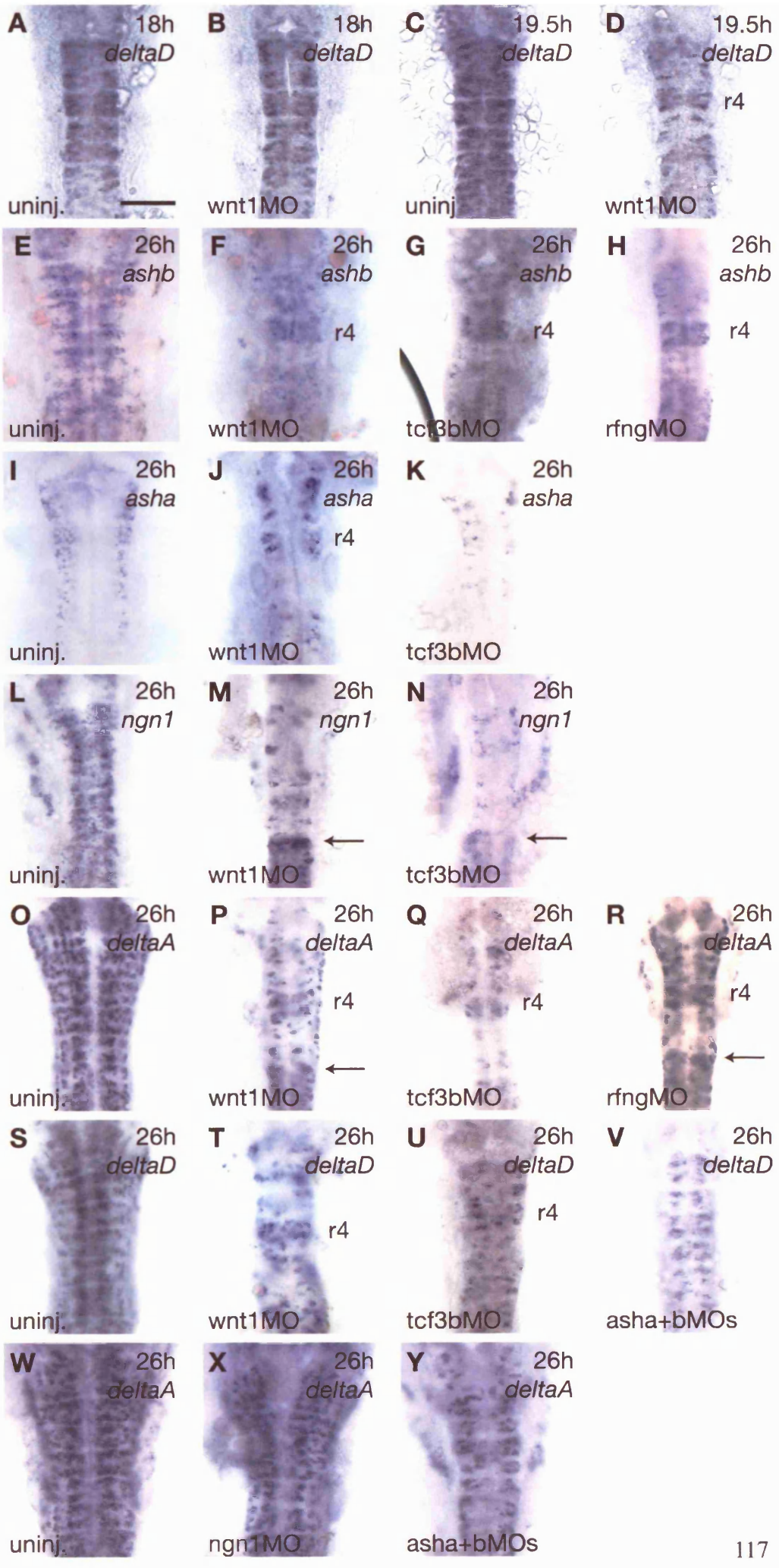
Analysis of *deltaD* expression in wnt1MO embryos reveals no apparent change in injected embryos at 18h compared to their uninjected siblings (Fig.15A,B), whereas at 19.5h, there is a major decrease in the number of cells expressing *deltaD* in the hindbrain (Fig.15C,D). The timing of this decrease in neurogenesis correlates with the timing of boundary spreading following *wnt1* knockdown (Fig.12A-D). This decrease is even more apparent by 24h, when few cells express *deltaD*, although r4 has a higher number of expressing cells than other rhombomeres (Fig.15S,T). Similar patterns are seen at 24h for *deltaA* and *deltaB* (Fig.15O,P, not shown for *deltaB*), when only a few expressing cells are left, except in r4, and the stripes of expression adjacent to boundaries in wild type embryos are absent. The same phenotype of a decreased number of Delta-expressing cells is seen in embryos injected with tcf3bMO, with fewer labelled cells compared with *wnt1* knockdowns (Fig.15Q,U). This suggests that Wnt

signalling through Tcf3b is required for *delta* gene expression. The relative contributions of boundary and non-boundary *wnt1* sources were assessed by comparing these phenotypes with those of embryos injected with rfngMO. Again, although the rfngMO phenotype is slightly milder than for wnt1MO injections, for all *delta* genes analysed, there is a large reduction in the number of expressing cells, with r4 being the least affected (Fig.15R). This indicates that boundary Wnt1 is required for normal levels of *delta* expression and neurogenesis in the hindbrain.

Knockdown of *wnt1* leads to decreased expression of the proneural genes, *asha* (Fig.15I,J), *ashb* (Fig.15E,F) and *ngn1* (Fig.15L,M). At 24h, two distinct levels of expression of *ashb* and *ngn1* can be distinguished : high levels of expression are present adjacent to boundaries and correspond to presumptive neuroblasts, and low levels of expression are found throughout the ventricular zone of rhombomere centres. In wnt1MO-injected embryos, both types of expression are decreased, and only scattered expressing cells remain. However, in the case of *ashb*, as for Delta genes, injected embryos have stronger expression in rhombomere 4. *asha* is expressed in scattered dorsal cells, but is excluded from boundaries. Expression in wnt1MO embryos is present in fewer cells than in uninjected siblings.

Fig. 15 : Regulation of proneural and delta genes by Wnt signalling.

A-D : time course of the effect of wnt1MO injection on *deltaD* expression. No effect is seen at 18h (A,B), but at 19.5h, *deltaD* is strongly downregulated (D). E-U : effect of knockdowns of *wnt1*, *tcf3b* and *rfng*, as indicated in the bottom left of each panel on proneural gene expression (E-H : *ashb*, I-K : *asha*, L-N : *ngn1*) and *deltaA* (O-R) and *deltaD* (S-U) expression. All are downregulated in injected embryos, but expression is often stronger in rhombomere 4 (r4), and posterior to the r6/r7 boundary (arrows in M,N,P,R). V-Y : knockdowns of proneural genes, as indicated at the bottom left of the panels causes a reduction in *deltaD* (V) and *deltaA* (W-Y) expression. All views are dorsal with anterior at the top. Scale bar : 100μm.


A 18h deltaD uninj.
B 18h deltaD wnt1MO
C 19.5h deltaD uninj
D 19.5h deltaD r4 wnt1MO
E 26h ashb uninj.
F 26h ashb r4 wnt1MO
G 26h ashb r4 tcf3bMO
H 26h ashb r4 rfngMO
I 26h asha uninj.
J 26h asha r4 wnt1MO
K 26h asha tcf3bMO
L 26h ngn1 uninj.
M 26h ngn1 wnt1MO
N 26h ngn1 tcf3bMO
O 26h deltaA uninj.
P 26h deltaA r4 wnt1MO
Q 26h deltaA r4 tcf3bMO
R 26h deltaA r4 rfngMO
S 26h deltaD uninj.
T 26h deltaD r4 wnt1MO
U 26h deltaD r4 tcf3bMO
V 26h deltaD asha+bMOs
W 26h deltaA uninj.
X 26h deltaA ngn1MO
Y 26h deltaA asha+bMOs

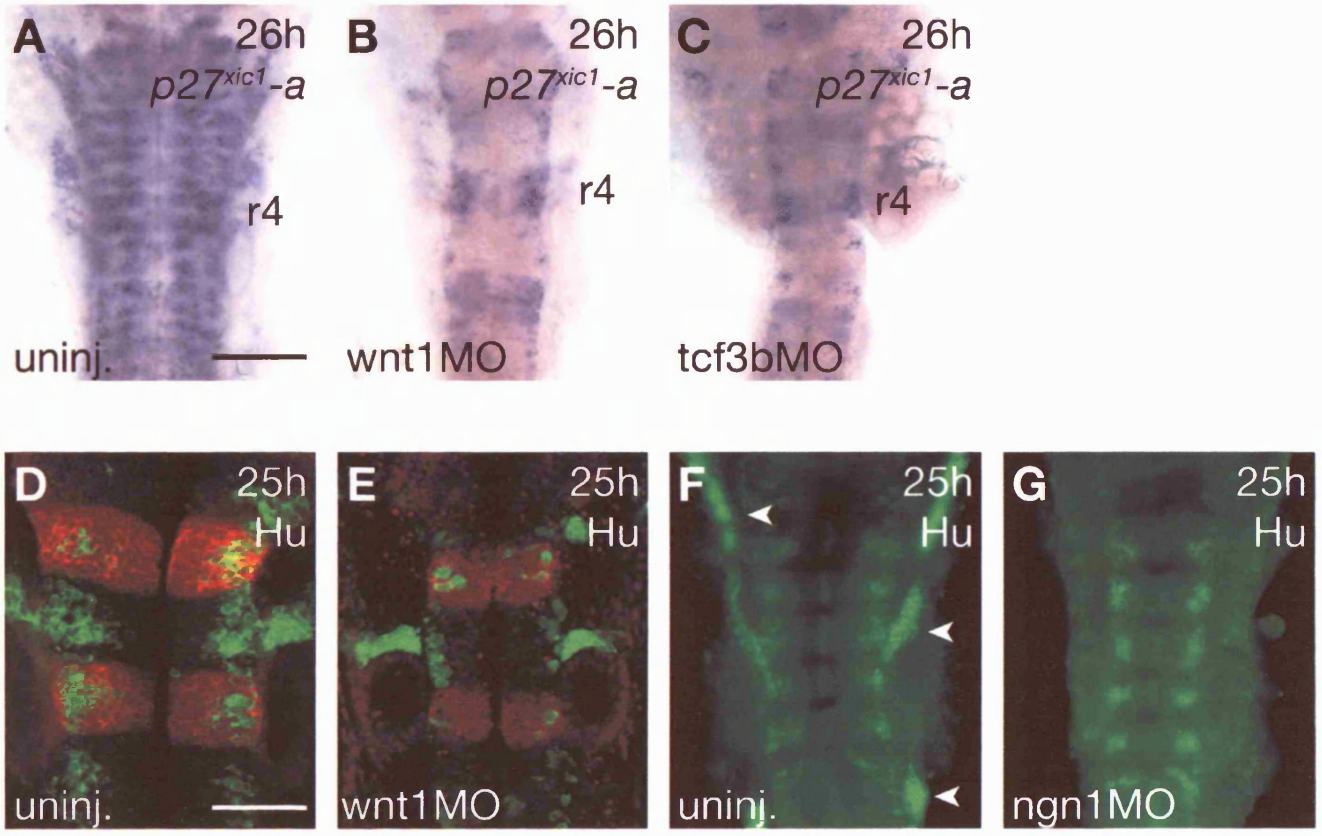
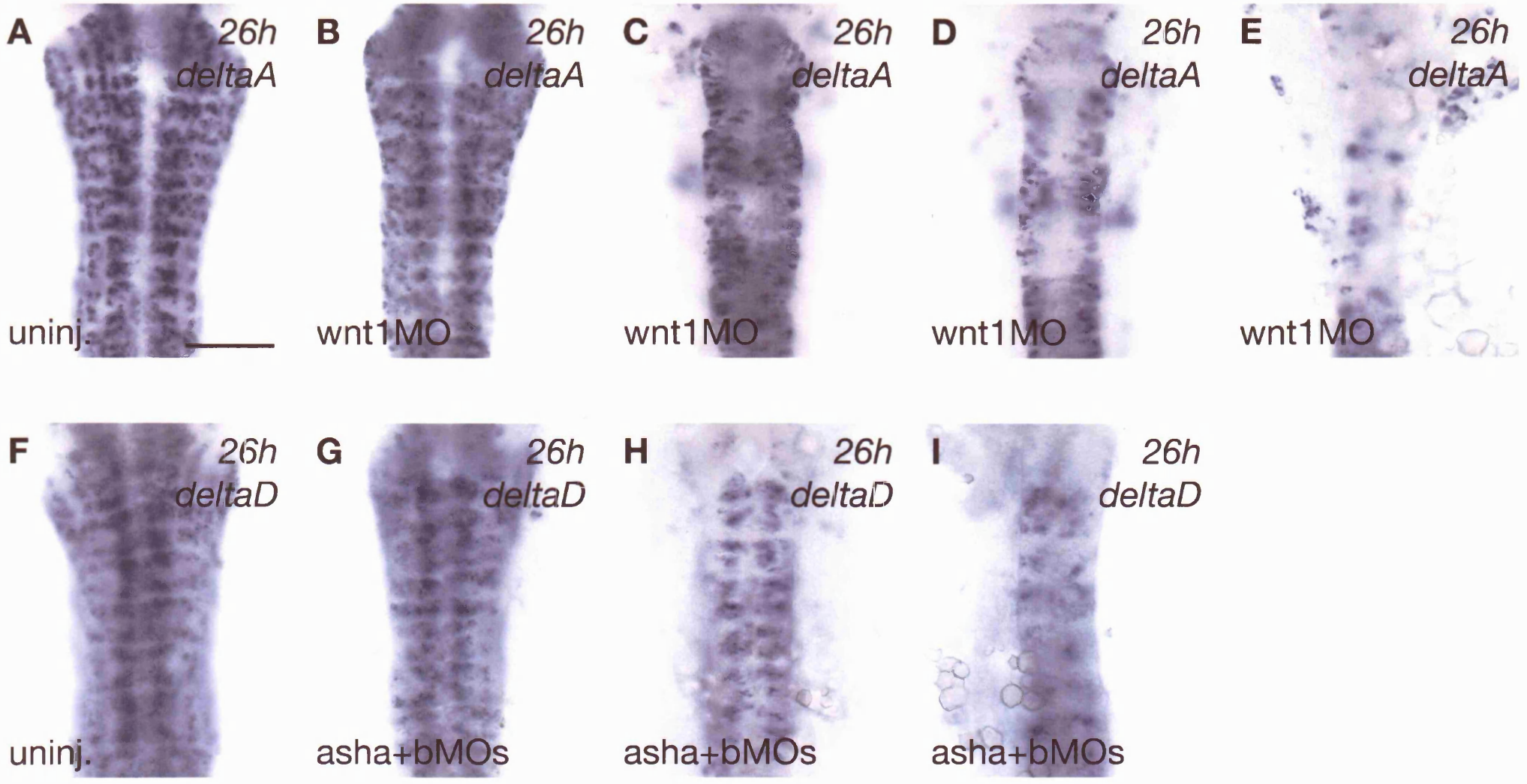


Fig. 16 : Neurogenesis is decreased following Wnt pathway or proneural gene knockdown.

Expression of p27xic1-a is decreased in wnt1MO (B) and tcf3bMO (C) embryos, compared to uninjected (A). Stronger expression is detected in rhombomere 4 (r4). D,E : immunohistochemical detection of Hu antigens (green) shows a strong decrease in the number of Hu-positive neurons in wnt1MO-injected embryos. EphA4 is shown in red to indicate rhombomeres 3 and 5. F,G : Hu immunohistochemistry shows that no neurons are present in the cranial ganglia of ngn1MO-injected embryos (G, arrowheads in F indicate cranial ganglia), and that there are fewer neurons within the hindbrain. Scale bar, A-C,F,G : 100μm ; D,E : 50μm.

Fig. 17 : Range of phenotypes of delta gene expression in wnt1MO or asha+bMO embryos.

A-E : *deltaA* expression in uninjected (A) or wnt1MO-injected embryos (B-E). The phenotypes were obtained in injected embryos with the following proportions : A, 0%; B, 3.5% (2/57 embryos); C, 25% (14/57 embryos); D, 58% (33/57); E, 14% (8/57 embryos). F-I : *deltaD* expression in uninjected (F) and embryos injected with both ashaMO and ashbMO (G-I). G-I are representative of the phenotypes observed, with 12.5% of embryos (2/16) resembling G, 69% (11/16) similar to H, and 19% (3/16) like I. Scale bar: 100μm.


A
26h
deltaA
uninj.
B
26h
deltaA
wnt1MO
C
26h
deltaA
wnt1MO
D
26h
deltaA
wnt1MO
E
26h
deltaA
wnt1MO
F
26h
deltaD
uninj.
G
26h
deltaD
asha+bMOs
H
26h
deltaD
asha+bMOs
I
26h
deltaD
asha+bMOs

Similar phenotypes are observed in embryos injected with tcf3bMO (Fig.15G,K,Q), suggesting that Wnt1 signalling through Tcf3b is required for proneural gene expression. Tcf3bMO embryos have fewer expressing cells in the hindbrain than wnt1MO embryos. Knockdown of *rfng* also causes a decrease in *ashb* expression (Fig.15H), but the phenotype is milder than for wnt1MO. As for *wnt1* and *tcf3b* knockdowns, injected embryos have low-level expression of *ashb* in rhombomere 4. It is also of note that, particularly for *ngn1* and Delta expression, a much weaker effect is observed caudal to rhombomere 6 (arrows in Fig.15M,N,P-R mark the posterior border of r6).

As previous studies of the regulation of *delta* gene transcription have shown that proneural genes are essential for *delta* expression (Haenlin et al., 1994; Hans and Campos-Ortega, 2002), an obvious prediction would be that the reduction of *delta* expression observed is due to the reduction of proneural gene expression. This idea is supported by knockdown experiments of either *asha*, *ashb* or *ngn1*, or combinations of knockdowns of these genes, in which *deltaA* and *deltaD* expression is reduced (Fig.15V-Y). These experiments do not preclude the possibility of a direct input of Wnt signalling into Delta expression, but they do suggest that Wnt1 affects expression of Delta genes via proneural genes.

The observation that both proneural and *delta* gene expression is decreased in wnt1MO embryos is indicative of a decrease in neurogenesis. I have therefore examined in more detail the distribution of markers of neurogenesis.

The expression of $p27^{Xic1}$-*a* reveals the cells that are leaving the cell cycle, and $p27^{Xic1}$-*a* is therefore a marker of differentiating cells. In one day-old embryos, $p27^{Xic1}$-*a* expression is strongly decreased in wnt1MO embryos

(Fig.16A,B), indicating that many fewer cells are leaving the cell cycle at this time point. Similarly, in *tcf3b* knockdowns, *p27^{Xic1}-a* expression is decreased (Fig.16C), suggesting that Wnt1 signals through Tcf3b to allow neuronal differentiation in the hindbrain. As noted previously with other markers, most labelled cells are found in r4 in the morphants. Detection of differentiated neurons with Hu antibody reveals the cumulative effect of this lack of differentiation, and indeed, there are fewer neurons at 26h in wnt1MO embryos, most of which are found ventrally in the centre of rhombomeres (Fig.16D,E). Knockdown of proneural genes also leads to reduced neurogenesis. In ngn1MO embryos, fewer Hu-positive cells are observed in the hindbrain, and, consistent with published data (Andermann et al., 2002), no neurons are present in the cranial ganglia of these morphants (Fig.16F,G, arrowheads point to Hu-positive cells in the cranial ganglia of uninjected siblings, which are absent in injected embryos).

Range of phenotypes in injected embryos.

As previously described for boundary markers, a range of phenotypes was observed with each morpholino for each marker. The images shown in Fig.15 correspond to the most commonly observed phenotype in each case. For instance, in the case of *deltaA* expression in wnt1MO-injected embryos, a continuum of phenotypes ranging from robust expression to almost no expressing cells was observed (Fig.17A-E). While no embryos have as strong expression as uninjected or control morpholino-injected embryos, 3.5% have stripes with fewer *deltaA*-expressing cells adjacent to boundaries (Fig.17B, 2/57 embryos, data from two separate experiments). A larger proportion, almost 25%

(14/57 embryos), does not display evidence of organisation of *deltaA*-positive cells into stripes, but expression is present throughout most of the hindbrain (Fig.17C). *deltaA* expression is weakest in rhombomeres 5 and 6. A stronger phenotype was observed in 58% of embryos (33/57), in which few cells in each rhombomere express *deltaA*, except in rhombomere 4, throughout which low levels of expression remain. In other rhombomeres, the labelled cells are lateral and express high amounts of transcript (Fig.17D). Finally, in the most extreme cases (14%, 8/57 embryos), very few cells express *deltaA*, even in rhombomere 4 (Fig.17E). It is interesting to note that, from this data, there appears to be different sensitivities of different rhombomeres to increasing levels of morpholino, with r5 and r6 being most sensitive, then r2 and r3, and r4 being least sensitive. This is consistent with the results observed following injection of morpholinos against *tcf3b* or *rfng*. For instance, in Fig.15R, expression of *deltaA* in a rfngMO-injected embryo is weakest in r5 and r6, intermediate in r2 and r3, and stronger in r4. Similar results are seen for *ashb* expression in wnt1MO, tcf3bMO or rfngMO embryos (Fig.15F-H).

Similarly, the pattern of *deltaD* expression in embryos injected with morpholinos against both *asha* and *ashb* displays some variability. No embryos have the same pattern as uninjected or control morpholino-injected embryos (Fig.17F). In 12.5% of embryos (2/16), high-level *deltaD* expression is present in stripes adjacent to boundaries in most rhombomeres, similar to, but weaker than in uninjected embryos (Fig.17G). Most embryos (69%, 11/16) have only a few *deltaD*-positive cells located ventrally in each rhombomere (Fig.17H). In the most severe cases, few cells express *deltaD*, although it is not completely absent

from the hindbrain (Fig.17I, 3/16 embryos, 19%). It seems likely that *ngn1* is responsible for the remaining *deltaD* expression.

In summary, some variability in the strength of the phenotype is seen for all morpholinos, and this may in part be due to variations in amount injected. However in all cases examined, the expression of proneural and Delta genes is reduced following knockdown of the Wnt pathway or of *rfng*, and Delta gene expression is reduced following proneural gene knockdown.

Analysis of patterns of differentiation in wnt1 *knockdowns at 24h and 48h.*

The stage-specific effect of *wnt1* knockdown implies that Wnt1 may be required only for neural cell types that differentiate after 18h of development. To analyse this, I have examined the expression of several cell type-specific markers at 48h where there is a well-characterised pattern of cell types (see results shown above and Trevarrow et al., 1990). Detection of a subset of reticulospinal neurons with RMO-44 antibody shows that these are still present in wnt1MO embryos, although axon pathfinding and fasciculation defects can be seen (Fig.18A,B). Cranial motor neurons, as detected by *in situ* hybridisation with *tbx20* probe, are present in wnt1MO embryos (Fig.18C,D), but there are fewer cells, particularly in the more posterior regions. However, this contrasts with the midbrain and rhombomere 1, where almost all motor neurons of the IIIrd and IVth nerves are absent, suggesting that the requirement for Wnt1 in the specification of cranial motor neurons differs between the midbrain-hindbrain area and the hindbrain itself.

At 48h, *dbx1* and *pax3* are expressed in stripes of neuronal cells, as well as in continuous columns in the ventricular zone (Fig.18E,G). In wnt1MO embryos, although there does not seem to be a major reduction in the number of cells labelled with either *dbx1a* or *pax3*, the pattern is disorganised, and no clear stripes of neuronal cells are visible (Fig.18F,H). From dorsal views, it appears that most staining for these two markers in wnt1MO embryos is ventricular. To analyse this, I detected *dbx1a* expression in conjunction with Hu staining in transverse sections (Fig.18I-N). The ventricular zone (VZ) is much thicker in wnt1MO embryos, and the proportion of Hu-labelled cells is greatly decreased. Most *dbx1a* expression is within this enlarged progenitor cell domain, and only few cells are found that are co-labelled with Hu. Finally, and in accordance with the known phenotype of loss of proneural genes (Nieto et al., 2001), there is an increase in *gfap* expression in the hindbrain of embryos in which *wnt1* has been knocked down (Fig.18O,P). The organisation of both the cell bodies and the fibres into stripes is also lost, and both RNA and protein can be detected at high levels throughout the hindbrain (Fig.18Q,R).

These phenotypes of selective loss of neuronal cell types are consistent with a requirement for Wnt1 for neurogenesis only subsequently to 18h of development, as suggested by the effects on *deltaD* expression. Reticulospinal neurons are mostly born before 15h, motor neurons are born between 16h-20h, and neurogenesis adjacent to boundaries takes place from about 22h (Chandrasekhar et al., 1997; Mendelson, 1986). Furthermore, the presence of an enlarged ventricular zone, and of ectopic *gfap*-expressing cells, demonstrates that lack of neurogenesis is not due to a loss of progenitors, rather, it reflects a block in neuronal differentiation.

Fig. 18 : Neural subtype specification in wnt1MO embryos.

Dorsal views of whole mount (A-H,O-R) and transverse cryostat sections (I-N) of 48h embryos. Reticulospinal neurons (RMO-44 immunohistochemistry, A,B) are normally specified and cranial motor neurons, though hypomorphic, are still present (*tbx20*, G,H). Stripes of *pax3* (E,F) and *dbx1a* (G,H) expression are disrupted. Sectioning reveals that most *dbx1a*-positive cells in wnt1MO embryos are non-neuronal ventricular zone cells (I,L, ventricular zone is determined by DAPI stain and Hu immunohistochemistry, J,K,M,N), and that there are much fewer neurons. O-R : analysis of *gfap* expression (O,P) and of protein distribution (Q,R), revealing excess of glial cell types and disorganisation of glial fibres. VZ : ventricular zone, MZ : mantle zone. Scale bar : 50μm in A-B,I-N and Q-R, 100μm in C-H and O,P.

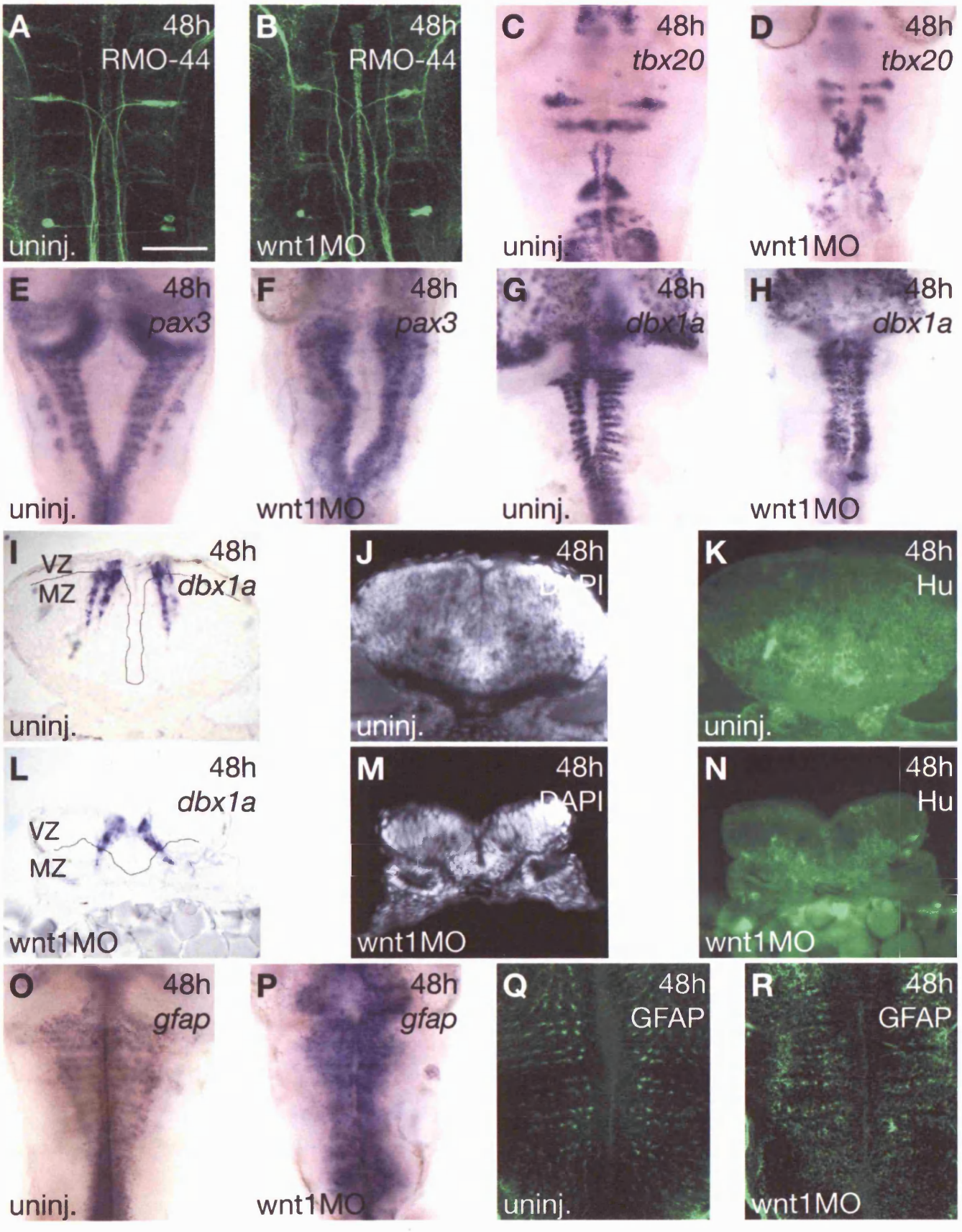
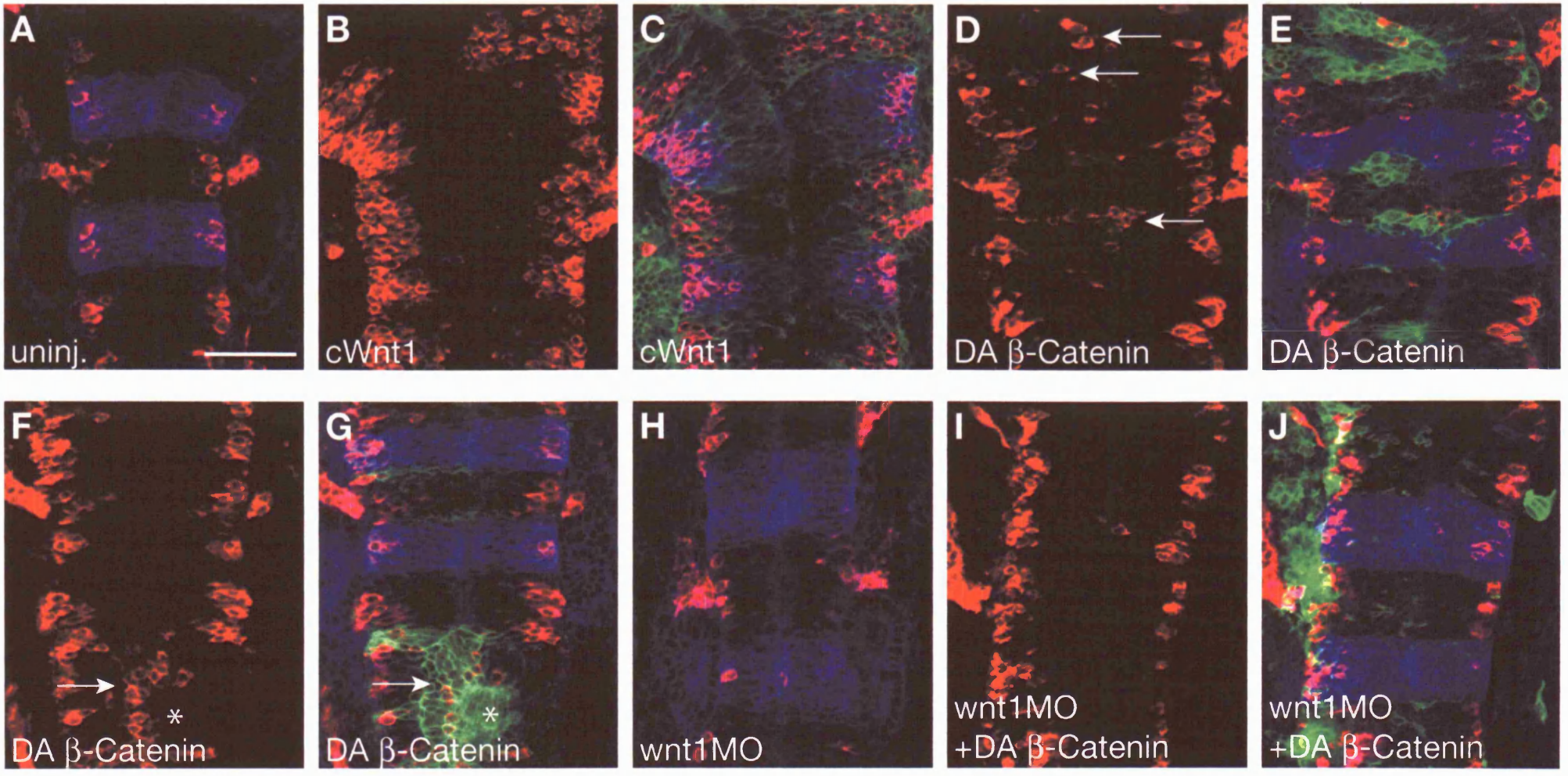

A
48h
RMO-44
uninj.
B
48h
RMO-44
wnt1MO
C
48h
tbx20
uninj.
D
48h
tbx20
wnt1MO
E
48h
pax3
uninj.
F
48h
pax3
wnt1MO
G
48h
dbx1a
uninj.
H
48h
dbx1a
wnt1MO
I
VZ
MZ
48h
dbx1a
uninj.
J
48h
uninj.
K
48h
Hu
uninj.
L
48h
dbx1a
VZ
MZ
wnt1MO
M
48h
wnt1MO
N
48h
Hu
wnt1MO
O
48h
gfap
uninj.
P
48h
gfap
wnt1MO
Q
48h
GFAP
uninj.
R
48h
GFAP
wnt1MO

Fig. 19 : Effect of Wnt pathway activation on neurogenesis.

HuC/HuD immunohistochemistry (red) showing the distribution of neurons in either uninjected (A), full length chick Wnt1-injected (B,C), dominant active β-catenin-injected (D-G), wnt1MO-injected (H) or embryos co-injected with wnt1MO in all cells and mosaic dominant active β-catenin (I,J). EphA4 is shown in blue to identify rhombomeres 3 and 5, while the distribution of the injected construct is shown by co-injection of GFP (green). B,C ; D,E and F,G show the same embryos, but in B,D,F only the red channel is shown for the sake of clarity. Overexpression of mosaic cWnt1 causes a general increase in neurogenesis (B,C), while cells expressing DA β-catenin often ectopically express Hu (arrows in D-G), although sometimes clusters of high-expressing cells form in which no Hu-positive cells can be seen (asterisk in F,G). DA β-catenin is able to rescue in part the loss of neurogenesis caused by injection of wnt1MO (H-J). Scale bar : 50μm.


A
uninj.
B
cWnt1
C
cWnt1
D
DA β-Catenin
E
DA β-Catenin
F
DA β-Catenin
G
DA β-Catenin
H
wnt1MO
I
wnt1MO
+DA β-Catenin
J
wnt1MO
+DA β-Catenin

Activation of the Wnt pathway can induce neurogenesis.

Studies in the chick spinal cord have shown that ectopic activation of the Wnt/β-Catenin pathway promotes cell proliferation and inhibits neuronal differentiation (Megason and McMahon, 2002). I have therefore tested whether Wnt1 promotes or inhibits neurogenesis in the zebrafish hindbrain by overexpressing either full length chick Wnt1 (cWnt1) or a stabilised form of β-Catenin, which lacks the first 87 amino acids, including the phosphorylation sites for GSK3 (Domingos et al., 2001).

Mosaic overexpression of cWnt1 leads to an increased number of neurons in the hindbrain compared to uninjected siblings (Fig.19B,C, compare with Fig.19A). Although it has not been quantified in these experiments, there does seem to be an increase in cell proliferation, as the hindbrain appears broader than in control embryos. Thus, although Wnt signalling may regulate proliferation, it does not appear to be inhibitory for neuronal differentiation in the hindbrain.

Confirmation of this result was obtained using an approach that allows to examine the cell autonomy of the phenotype. By mosaically co-injecting a dominant active form of β-Catenin with green fluorescent protein, one can determine whether cells in which the Wnt signalling pathway is continuously activated are inhibited from becoming neurons, as shown in the chick (Megason and McMahon, 2002). However, β-Catenin-expressing ectopic neurons were observed at aberrant locations, such as the ventricular surface, in the hindbrain of embryos injected with these RNAs (Fig.19D-G). This finding suggests that continuous exposure to Wnt signalling is permissive for neurogenesis, and may actively promote neuronal differentiation. However, very high levels of expression do seem to be inhibitory, as the stronger-expressing cells often form

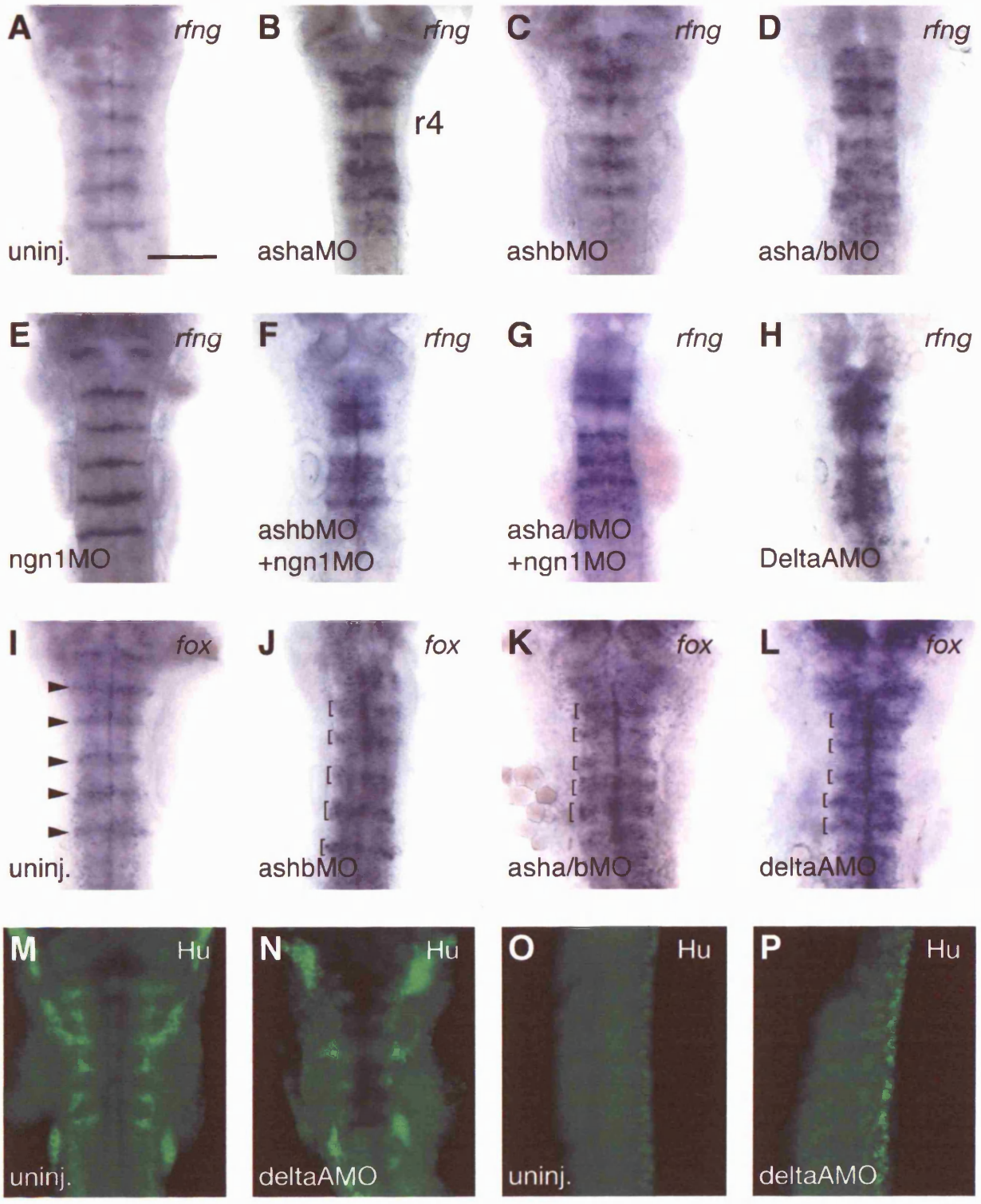
clumps which do not contain any Hu-positive cells, whereas cells with lower expression levels do express Hu, as seen in the posterior hindbrain of the embryo shown in Fig.19F,G. Finally, overexpression of stabilised β-Catenin in wnt1MO embryos can rescue the decrease in neurogenesis (compare the left and right sides of the embryo shown in Fig.19I,J). Since activating a downstream mediator of Wnt signalling can rescue the lack of Wnt1, this argues both for a requirement for Wnt/β-Catenin signalling in neurogenesis, and for the specificity of the neuronal phenotype of the *wnt1* morpholino.

Proneural genes and deltaA *repress boundary markers.*

Having shown that Wnt1 regulates expression of proneural genes and of delta genes in the hindbrain, I next tested whether these latter genes have a role in preventing the expansion of boundary markers. Knockdown of *asha* leads to expansion of both *rfng* and *foxb1.2* boundary markers (Fig.20B, *foxb1.2* not shown), as does blocking *ashb* translation, although to a lesser extent (Fig.20C,J). By contrast, in *ngn1* morpholino-injected embryos, little, if any boundary marker expansion is seen (Fig.20E), yet ngn1MO and ashbMO double knockdowns synergise to give a stronger expansion phenotype than either morpholino alone (Fig.20F). Similarly, *asha* and *ashb* double knockdowns also give a stronger phenotype than single knockdowns (Fig.20D,K).

Fig. 20 : Proneural and delta genes regulate boundary expansion.

A-H : *rfng* expression in either uninjected (A) or in single or combined knockdowns of *asha*, *ashb* and *ngn1* (B-G) or in *deltaA* knockdowns, all shown in dorsal views at 24h, in which expansion occurs. I-L : *foxb1.2* expression, arrowheads in L point to the sharp boundary expression domain, which is broader in knockdowns of proneural genes or of *deltaA* (brackets in J-L). M-P : Hu immunohistochemistry on a deltaAMO embryo in which *rfng* expression is seen to expand, showing decreased neurogenesis in the hindbrain (M,N), whilst it is increased in the spinal cord (O,P). Scale bar : 100μm.


A
rfng
uninj.
B
rfng
r4
ashaMO
C
rfng
ashbMO
D
rfng
asha/bMO
E
rfng
ngn1MO
F
rfng
ashbMO
+ngn1MO
G
rfng
asha/bMO
+ngn1MO
H
rfng
DeltaAMO
I
fox
uninj.
J
fox
ashbMO
K
fox
asha/bMO
L
fox
deltaAMO
M
Hu
uninj.
N
Hu
deltaAMO
O
Hu
uninj.
P
Hu
deltaAMO

An additional question that can be addressed is whether the absence of boundary marker spreading into r4 is due to the continued expression of proneural genes in this rhombomere in *wnt1*, *tcf3b* or *rfng* knockdowns. However, in a triple knockdown of *asha*, *ashb* and *ngn1*, *rfng* expression is still excluded from r4 (Fig.20G), indicating that another mechanism is responsible for preventing boundary fate acquisition within r4. There may be other, unknown, proneural genes, but this is unlikely as almost no neurons were detected in the hindbrains of embryos in which *asha*, *ashb* and *ngn1* have been knocked down (not shown).

Finally, the role of delta genes was addressed, particularly that of *deltaA* and *deltaD*, as their expression patterns suggest that they are the most relevant, *deltaB* being found exclusively in maturing neuroblast, outside the ventricular zone. In the *deltaD* mutant, *after eight*, no expansion of *rfng* expression was detected (not shown), however in *deltaA* knockdowns, ectopic boundary markers can be seen throughout the hindbrain, with the exception of r4 (Fig.20H). Due to the role of Delta genes in lateral inhibition, *deltaA* knockdown might be predicted to increase the amount of neurogenesis (Chitnis et al., 1995; Haddon et al., 1998; Holley et al., 2000). Indeed, in the spinal cord there are more Rohon-Beard neurons following *deltaA* knockdown (Fig.20O,P). In contrast, there is decreased neurogenesis in the hindbrain in deltaAMO embryos (Fig.20M,N), and this is consistent with a role of DeltaA in the boundary regulation of neurogenesis.

Conclusions.

The data presented in the previous sections provide support for a model in which Wnt1 signal from boundaries acts via Tcf3b to promote proneural and delta gene expression, and therefore neurogenesis in non-boundary cells, thereby preventing them from adopting a boundary fate.

Discussion.

In this work, I have identified a role of Wnt1 in regulating neurogenesis and preventing the spread of boundaries. The wider implications of the findings and models of boundary and hindbrain development will be discussed later (see "discussion and perspectives"). Here, I will discuss how this work relates to previous studies of the roles of Wnts in proliferation and neurogenesis, and the similarities between the genetic pathway in the zebrafish hindbrain and in the formation of sensory hair cells in the *Drosophila* wing imaginal disc.

Proliferation versus neurogenesis.

A role of Wnt signalling in proliferation.

Many studies have implicated Wnt/β-catenin signalling in the positive regulation of cell proliferation, both during development of neural tissues (Chenn and Walsh, 2002; Dickinson et al., 1994; Megason and McMahon, 2002; Panhuysen et al., 2004; Zechner et al., 2003) and, when deregulated, in cancer (reviewed in Giles et al., 2003). In addition, a number of these studies found that Wnt or β-catenin gain of function leads to a decrease in neurogenesis and a concomitant increase in progenitors re-entering the cell cycle (Chenn and Walsh, 2002; Megason and McMahon, 2002; Zechner et al., 2003). Wnt targets, including *cyclinD1* and *cyclinD2* are expressed in the spinal cord in response to Wnt signalling and mediate at least part of the mitogenic effect of Wnts (Megason and McMahon, 2002). These studies lead to the conclusions that Wnt signalling increases neural progenitor numbers by reducing their differentiation rate as well as shortening the cell cycle. Moreover, in β-catenin loss of function experiments, the differentiation rate is greatly increased and progenitors are not maintained (Zechner et al., 2003).

In the experiments I have carried out, knockdown of *wnt1* is accompanied by a decrease in cell proliferation in the hindbrain (Fig.14G). Furthermore, the hindbrains of embryos injected with full length *wnt1* RNA are broader than those of uninjected siblings (Fig.19A-C). These findings indicate that Wnt signalling does promote cell proliferation in the zebrafish hindbrain, as in other systems. Moreover, the fact that early differentiation occurs primarily in rhombomere centres (Fig.6C and Hanneman et al., 1988) suggests similarities to the spinal

cord where neurogenesis initially occurs distant from the Wnt source. However, I find that after 18h of development, Wnt1 is required for neurogenesis, and that overexpression of *wnt1* does not suppress neurogenesis, but rather increases the number of neurons (Fig.19A-C).

Proliferation defects cannot account for the phenotypes observed.

One interpretation of these findings could be that Wnt1 is selectively required for the proliferation of progenitors of neuronal cell types born after 18h, and that these progenitors are depleted in *wnt1* morphants. However, the total decrease in cell number at 19h is only approximately 15%, suggesting this may not account for the greater decrease in neurogenesis. Furthermore, while there is a decrease in proliferation in wnt1MO embryos, this affects non-boundary cells less than boundary cells, and yet most cells in r2, r3, r5 and r6 up-regulate hindbrain boundary markers. This indicates a change of fate, rather than a simple role in selectively maintaining a group of progenitors. Further evidence is given by the fact that progenitors expressing *dbx1a* are still present (Fig.18P), and accumulate in the enlarged ventricular zone. This shows that the decreased neurogenesis is due to a block in differentiation, not a loss of progenitors. Moreover, the excess *gfap* expression in wnt1MO embryos is consistent both with a block in neuronal differentiation (Nieto et al., 2001) and with a change of fate of cells to adopt a boundary identity. Therefore, although cell proliferation is affected in *wnt1* knockdowns, the decrease in neurogenesis is due mainly to a switch of progenitors from non-boundary to boundary identity. As boundaries are progenitors that do not express proneural genes (see Fig.7), they generate few neurons, if any.

Can different models be reconciled ?

Although the knockdown experiments of *wnt1* in the zebrafish hindbrain agree with a role for Wnt signalling in the regulation of proliferation, the observation that neurogenesis depends on the presence of Wnt1 appears contradictory to previous models of a role of Wnts in suppressing neurogenesis. However, there are many inconsistencies in the literature about the effect of overexpressing Wnts or β-catenin. In transgenic mouse embryos expressing *wnt1* under the control of the *hoxb4* promoter, dorsal parts of the spinal cord are preferentially expanded without any apparent inhibition of neurogenesis (Dickinson et al., 1994). This role in the regulation of dorsal proliferation is consistent with the observation that the dorsal hindbrain is reduced in *wnt1/wnt3a* double mutants (Ikeya et al., 1997). However, in chick embryos electroporated with an expression construct for *wnt1*, proliferation is increased only in ventral parts, while *β-catenin* overexpression affects all dorso-ventral levels (Megason and McMahon, 2002). Moreover, in the midbrain-hindbrain area, overexpression of *wnt1* by different methods also yields different results. In transgenic mouse embryos ectopically expressing *wnt1* under the control of the *en1* promoter, increased proliferation is seen only in one midbrain structure, the inferior colliculi, and only at specific stages, although *cyclinD1* is upregulated until adulthood in these embryos (Panhuysen et al., 2004). Interestingly, in these embryos, there is a slight shift in the relative densities of neurons and glia towards neurons, although total cell density is unchanged. Retroviral transfection of *wnt1* in chick embryos leads to increased forebrain size, but no effect is observed in the midbrain (Adams et al., 2000), while electroporation of *wnt1*

leads to a mild expansion of the tectum as well as the telencephalon, due to a transient increase in proliferation (Matsunaga et al., 2002). Finally, recent studies in the mouse neocortex and *in vitro* cell culture have shown that Wnt/β-catenin signalling can induce neurogenesis in a developmental context (Chenn and Walsh, 2003; Hirabayashi et al., 2004; Israsena et al., 2004; Kan et al., 2004; Muroyama et al., 2004; Otero et al., 2004) and in P19 cells (Lyu et al., 2003; Tang et al., 2002).

These apparent contradictions suggest that Wnt signalling may have different effects in different areas of the neural tube. Indeed, analysis of *ngn1* expression (Fig.15M) provides evidence of spatial differences in the response to *wnt1* knockdown in the zebrafish. Expression is strongly reduced in the hindbrain, but caudal to the r6/r7 boundary, little effect is observed. Moreover, the spatial relationship between *wnt1* and *cyclinD1* is different in the spinal cord and in the hindbrain. In the spinal cord, *cyclinD1* is expressed in a dorsal to ventral gradient, but not at high levels in the dorsal Wnt source (Megason and McMahon, 2002), but in the hindbrain transcripts are found at high levels in boundary cells. As well as this, there are differences in the way different cranial nerve neurons are affected. In the midbrain-hindbrain boundary area, the IIIrd and IVth nerves are not formed in wnt1MO embryos, while in the hindbrain itself, the cranial motor neurons form, although in slightly smaller numbers than uninjected siblings. This result is interesting in itself, as no published work has involved Wnt signalling in regulating the formation of cranial motor nuclei around the isthmus, especially as in *wnt1* mutants, the whole area is missing. In zebrafish embryos, knockdown of *wnt1* does not lead to loss of the midbrain or of midbrain-hindbrain boundary (MHB) markers (data not shown, but see Lekven et

al., 2003), suggesting that the loss of the IIIrd and IVth cranial nerves reflects a specific role of Wnt1 in neurogenesis rather than a general role in maintaining the MHB or midbrain. This result shows clearly that even a related population of neurons (cranial motor neurons in this instance) respond differently to the same signal according to their position along the anterior-posterior axis.

Another possibility is that Wnt signalling may have different roles at different times in development, as suggested by the stage-specific response of the tectum to ectopic Wnt1 (no increase in cell proliferation is observed before E11.5, although the transgene used is active by E9.5, Panhuysen et al., 2004). This possibility is again supported by the data obtained here, as no effect of the Wnt1 morpholino on neurogenesis or boundary spreading is observed before 18h in the hindbrain (Fig.15A,B). This idea has been proposed in a recent study in the mouse neocortex which has shown that Wnt/β-catenin signalling promotes neuronal differentiation of progenitors (Hirabayashi et al., 2004). This increased differentiation is observed with cells from embryonic day 13.5 (E13.5) embryos, but not with cells prepared from E10.5 embryos. Moreover, these and other authors show that a β-catenin/TCF complex can bind the *ngn1* promoter and activate transcription of this gene (Hirabayashi et al., 2004; Israsena et al., 2004).

A possible explanation for differences in response to Wnt signalling in different tissues and in the same tissue at different stages is that other factors are involved in modifying the response to Wnts. Fibroblast Growth Factors (FGFs) are good candidates for this role, as it has been shown that in the presence of FGF2, ectopic β-catenin leads to increased proliferation, but in the absence of FGF2, β-catenin promotes increased differentiation (Israsena et al., 2004). Another intriguing observation is that in gain and loss of function experiments

with β-catenin, while the rate of differentiation relative to the total number of cells is strongly affected, the absolute number of cells that differentiate over a period of time is the same (Zechner et al., 2003). This may simply be due to inhibition of differentiation by β-catenin, but may also indicate that another factor is limiting for differentiation, even when increased numbers of progenitors are present.

Finally, another explanation for the differences observed in different studies of Wnt/β-catenin signalling and its relationship to neuronal differentiation could be that different levels of signalling elicit different responses. Evidence of this is found by comparing the effect of retrovirus-induced misexpression and of electroporation of *wnt1* on proliferation in the tectum (Adams et al., 2000; Matsunaga et al., 2002). While the levels of transcription obtained from retroviral vectors do not lead to ectopic proliferation, electroporation of an expression vector, which leads to higher levels of expression, significantly increases BrdU incorporation in the tectum. Similarly, my experiments provide some evidence for differing effects according to levels of expression of dominant active β-catenin. I find that when high levels of GFP are visible, indicating a high amount of injected GFP and β-catenin RNA, they tend to be in clusters within which no cells are positive for Hu, and that ectopic Hu-positive cells have lower levels of GFP fluorescence. Finally, papers by Chenn and Walsh (2002; 2003) show that transgenic mouse embryos expressing different levels of dominant active β-catenin have different phenotypes. In embryos expressing high levels, proliferation is greatly increased and more cells re-enter the cell cycle rather than differentiate. In embryos expressing lower

levels, the increase in proliferation is more modest, and many ectopic neurons are seen (Chenn and Walsh, 2002; Chenn and Walsh, 2003).

It is therefore likely that the effects of Wnt signalling on neuronal differentiation are context-dependent, varying with time, levels of signal, and between different tissues. Moreover, Wnt signalling cannot always be interpreted as simply affecting the choice between proliferation and differentiation. For instance, in the *wnt1/wnt3a* double knockout, the two most dorsal neuronal types are very reduced, while a more ventral type is expanded, suggesting a role in choice of cell type fate rather than in regulating proliferation (Muroyama et al., 2002). As zebrafish rhombomere boundaries do not express proneural genes, it is difficult in this case to distinguish between a regulation of boundary versus non-boundary fate by Wnt1 in non-boundary cells and the induction of neurogenesis, as the two processes are linked.

Is Wnt1 instructive for neuronal differentiation ?

A question that is raised from these observations is whether Wnt1 is sufficient to instruct progenitors to differentiate or is a permissive factor. A clue comes from the observation that in wnt1MO or tcf3bMO embryos both low and high levels of proneural and delta gene expression are lost in the hindbrain, with the exception of r4 (see Figs.10 and 11). Studies of neurogenesis in *Drosophila* (Skeath and Carroll, 1994) and zebrafish (Haddon et al., 1998) have suggested that low-level proneural and Delta gene expression underlies a competence to differentiate, and that higher level expression occurs during differentiation. In the hindbrain, *ashb* and *ngn1* proneural genes, as well as *deltaA* and *deltaD* show

these two different expression levels, while expression of *p27^{Xic1}-a* and *deltaB* is restricted to the differentiating neuroblasts (Fig.7 and Haddon et al., 1998). The decrease in low-level proneural gene expression in wnt1MO embryos may therefore indicate that Wnt1 has a permissive role in neuronal differentiation.

That more neurons are observed when *wnt1* is ectopically expressed may simply be due to the fact that there is more proliferation in the injected embryos, and a consequent increase in the number of neurons. However, the presence of neurons at ectopic locations among cells expressing dominant active β-catenin indicates that activation of the Wnt pathway can lead to ectopic differentiation. This is consistent with a number of recent studies in other systems, showing in particular that Wnt/β-catenin activates transcription of the *neurogenin1* gene in mouse and causes stem cells to differentiate (Hirabayashi et al., 2004; Israsena et al., 2004; Muroyama et al., 2004). In this model, Wnt signalling leads directly to neuronal differentiation, and the stripes of differentiation adjacent to boundaries are due to high levels of secreted Wnt ligand being present there. Another way in which Wnt1 may influence the distribution of neurogenesis in the zebrafish hindbrain is by biasing Delta/Notch signalling such that high levels of delta genes are expressed adjacent to boundaries, such that other cells within hindbrain centres, although competent to differentiate, do not, as they are inhibited by Notch. This idea is supported by recent work showing that many consensus Tcf binding sites are present on the mouse *delta1* promoter (Galceran et al., 2004; Hofmann et al., 2004), including within the region of high homology with the zebrafish *deltaD* promoter, which drives expression within the nervous system (Beckers et al., 2000; Hofmann et al., 2004). In this model, Wnt1 signals throughout hindbrain segments for cells to express proneural genes, and then

high levels of Wnt1 bias the lateral inhibition process, causing differentiation to occur adjacent to boundaries.

Nevertheless, other indications suggest that Wnt signalling may not be organising the stripes of differentiation adjacent to boundaries. First, if high levels of Wnt1 cause differentiation in adjacent cells, one would expect differentiation to take place also adjacent to the roof plate, but this is not the case. Second, the expression of *wnt1* in boundaries is confined to the dorsal half of boundaries, but the stripes of differentiation occur adjacent to most of the dorso-ventral extent of boundaries (see for instance Fig.7O). Finally, by 24h, *wnt1* expression becomes increasingly dorsally restricted at boundaries, while neurogenesis adjacent to boundaries continues well beyond this stage.

Thus it seems likely that Wnt signalling is required for non-boundary cells to express uniform low levels of proneural genes and thus confer competency to differentiate to these cells (similar to the "equivalence groups" in *Drosophila*, where all cells expressing AS-C genes are competent to become neuronal progenitors, reviewed in Skeath and Carroll, 1994). Other factors are then required to cooperate with Wnt1 to instruct neuronal differentiation and organise the stripes of differentiation.

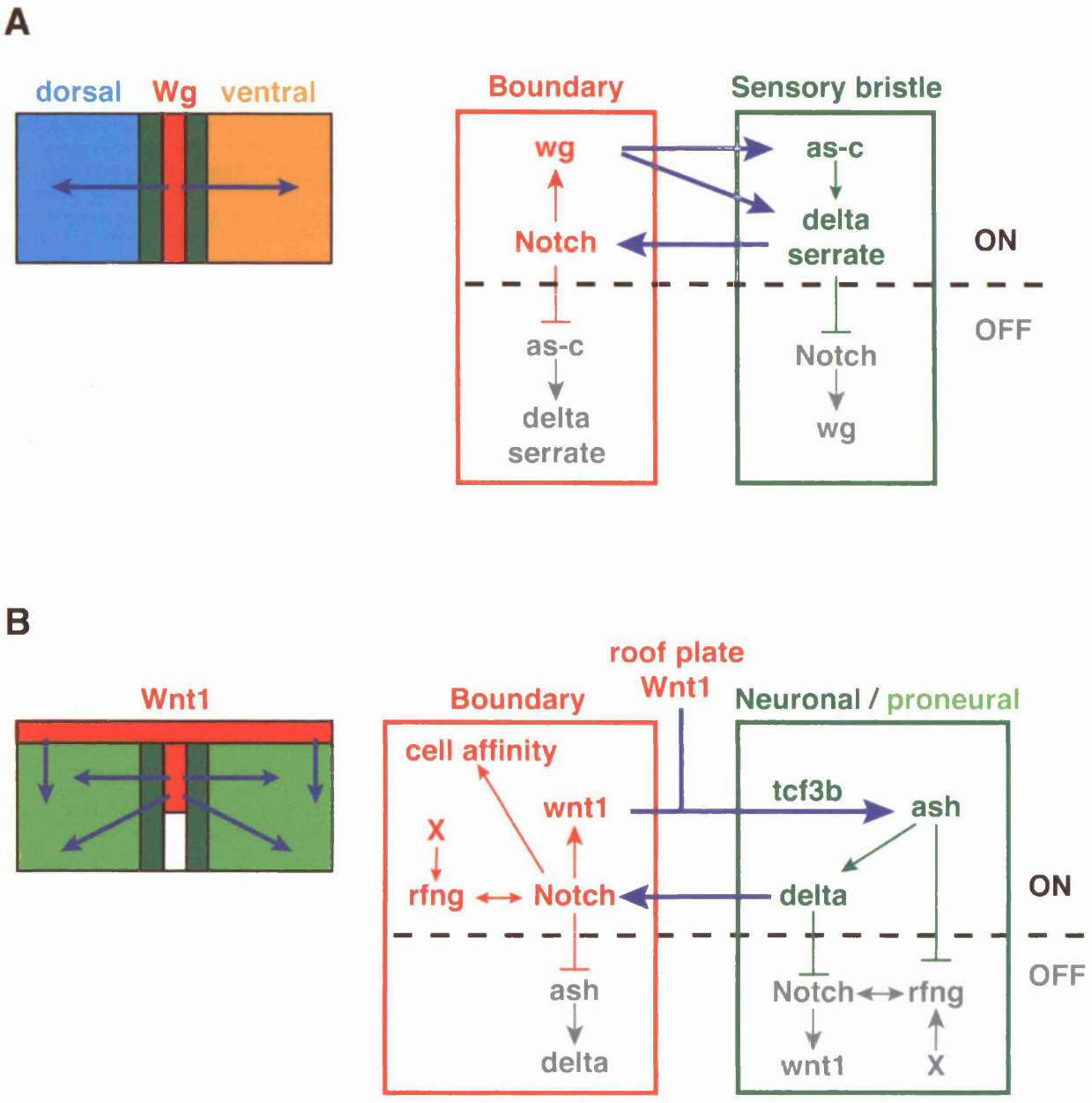


Fig. 21 : Model of regulation of cell differentiation and restriction of boundary spreading.

These diagrams illustrate the similarity between the regulatory gene hierarchies in the anterior compartment of the Drosophila wing imaginal disc (A), and the zebrafish hindbrain (B). In both systems, fringe-mediated modulation of Notch at the boundary causes expression of wg/wnt1. Wg/Wnt1 acts on neighbouring cells to upregulate proneural gene expression (as-c/ash), inducing neural fate. In Drosophila, Wg upregulates delta expression in neighbouring cells, but in vertebrates, delta genes are upregulated through proneural genes. Delta activates Notch in boundary cells, thus creating a feedback loop. In addition, proneural gene products and/or Delta act cell autonomously to prevent Notch activation and suppress boundary formation. In the zebrafish (B), Notch is not sufficient to induce boundary cell markers (Cheng et al., 2004), therefore another factor ('X') is proposed to be required for boundary cell specification.

A conserved gene network regulates neural differentiation and lateral inhibition.

Similarities between spatial relationships of gene expression in the hindbrain and in the wing imaginal disc of *Drosophila* suggested a model for the action of Wnt1 in the zebrafish hindbrain (Fig.21). I will begin by describing current understanding of the patterning of the wing blade in the fly, and I will discuss the evidence supporting this model in the zebrafish hindbrain, and the aspects of this model that differ between the zebrafish hindbrain and the *Drosophila* wing disc.

Boundary restriction and sensory organ differentiation in the fly wing.

In the wing imaginal disc of *Drosophila*, the Notch ligands *delta* and *serrate* are initially expressed in the ventral and dorsal compartments, respectively. Expression of *fringe* in the dorsal compartment renders those cells less responsive to *serrate* and more sensitive to signal from *delta*-expressing cells, such that a stripe of cells has elevated Notch signalling at the dorso-ventral boundary (Diaz-Benjumea and Cohen, 1995; Irvine and Wieschaus, 1994; Panin et al., 1997). Notch activation leads to expression of *wingless* in the boundary cells (de Celis and Bray, 1997; de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; Kim et al., 1995; Micchelli et al., 1997; Rulifson and Blair, 1995). Wingless then acts on adjacent cells, causing them to express both *delta* and *serrate* at high levels (de Celis and Bray, 1997; Micchelli et al., 1997; Yan et al., 2004), while expression of *cut* in boundary cells causes them to downregulate the Notch ligands (de Celis and Bray, 1997). In the anterior

compartment, Wingless regulates bristle formation along the wing margin by upregulating proneural genes *achaete* and *scute* in the adjacent cells (Blair, 1992; Blair, 1994; Couso et al., 1994; Johnston and Edgar, 1998; Neumann and Cohen, 1997; Phillips and Whittle, 1993; Rulifson et al., 1996; Simpson et al., 1988; Skeath and Carroll, 1991), and these genes also regulate *delta* expression (Haenlin et al., 1994). High levels of *delta* and *serrate* have a cell autonomous inhibitory effect on Notch activation and therefore the cells adjacent to boundaries cannot express *cut* or *wingless*, and consequently Wingless mediates a lateral inhibition that prevents spreading of its own expression domain (de Celis and Bray, 1997; de Celis and Bray, 2000; de Celis and Garcia-Bellido, 1994; Micchelli et al., 1997; Rulifson et al., 1996). Thus, patterning of sensory bristles is tightly linked to the regulation of the dorso-ventral boundary, maintaining a sharp signalling source (see Fig.21A).

A model for hindbrain patterning in the zebrafish.

In the zebrafish hindbrain, there is a remarkable similarity in the spatial relationships of expression patterns with the *Drosophila* wing imaginal disc, and the regulatory hierarchy of the homologous genes appears comparable (Fig.21). At early stages, *deltaA* and *deltaD* are expressed throughout rhombomeres, but are excluded from boundaries. The Notch pathway component *rfng* is expressed in boundaries, and there is indirect evidence that Rfng promotes Notch activation, as *wnt1* expression in boundaries is dependent on Rfng (this study and Cheng et al., 2004). Notch signalling does occur at boundaries and is sufficient to regulate the affinity properties of boundary cells (Cheng et al., 2004). After 18h of development, expression of proneural genes and of delta genes becomes

restricted to stripes adjacent to boundary cells. Knockdown of *wnt1* or *tcf3b* leads to a strong reduction in the number of cells expressing proneural and delta genes, and an expansion of the domain of expression of boundary-specific genes. Delta genes are under transcriptional control of proneural genes, as has been previously described in the case of *deltaD* (Hans and Campos-Ortega, 2002). Finally, knockdown of *asha*, *ashb*, *ngn1* or *deltaA* leads to ectopic boundary marker expression in the hindbrain, implicating these genes in the repression of boundary fate.

Thus Wnt1 regulates the boundary domain in a mechanism linking neuronal differentiation to maintenance of Notch activation in boundaries. This mechanism inhibits neighbouring cells from adopting a boundary fate, and has remarkable similarities to the genetic network between *notch*, *wingless*, *delta* and proneural genes in the *Drosophila* wing imaginal disc.

A recent paper, published while this work was being completed, also examines the roles of Wnt genes in patterning the zebrafish hindbrain (Riley et al., 2004). In this study, the authors find that four Wnt genes are expressed at boundaries (*wnt1*, *wnt3a*, *wnt8b* and *wnt10b*), and that they organise the stripes of *deltaA* expression, without affecting the total number of cells expressing *deltaA*. They interpret the phenotype in embryos deficient for all four Wnts (using a deletion that removes both *wnt1* and *wnt10b* as well as morpholinos against *wnt3a* and *wnt8b*) as a loss of rhombomere boundaries, based on the loss of organisation of the radial glial fibres and an apparent absence of boundary commissural neurons. Moreover, based on the previous interpretation of the change in *foxb1.2* expression in *tcf3b* morphants (Dorsky et al., 2003), the authors attribute the disorganisation of the hindbrain to a loss of boundaries, and

interpret the reduced *deltaA* expression as due to Wnt-independent roles of Tcf3b. The apparently fundamental difference in interpretation between that work and this may be explained simply by a lack of appropriate markers, as interpreting the phenotype of *foxb1.2* is difficult, due to its expression in the ventricular zone as well as in boundaries. Consequently, apparently uniform expression of *foxb1.2* was interpreted as being due to normal non-boundary expression, rather than a spreading of boundary expression. In contrast, analysis of *rfng* expression shows that boundaries have indeed expanded.

A more important question arising from Riley *et al.*'s work is how to account for the different effects on *deltaA* expression. While I found that in wnt1MO embryos, *deltaA* expression is strongly reduced (Fig.15P), a phenotype also observed in tcf3bMO embryos (Fig.15Q and Fig.3B in Riley et al., 2004), in embryos with a deletion spanning the *wnt1* and *wnt10b* loci, no apparent change in *deltaA* expression is seen (Fig.2A in Riley et al., 2004). The authors show that two more Wnts, Wnt3a and Wnt8b, need to be impaired for a strong effect to be observed. This is all the more surprising as I have shown that boundary expression of *wnt8b* is not upregulated in wnt1MO embryos, although I have not analysed *wnt3a* and *wnt10b* expression. One potential explanation is that the deletion which removes *wnt1* and *wnt10b* is larger than previously estimated (by using genetic distances, the authors estimate the deletion spans approximately 500kb Lekven et al., 2003), as the physical distance between the two markers that are deleted is 4Mb according to the current, although incomplete, genome assembly (Birney et al., 2004). Allowing for errors in the current assembly, a conservative estimate of the deletion size is approximately 1.5Mb. Moreover, many known and predicted transcripts are within this area, including at least the

first exon of a predicted homologue of *deltex3* (Curwen et al., 2004), whose activity inhibits neuronal differentiation in mouse and *Xenopus* (Kishi et al., 2001). Therefore, at least one of the genes that are deleted along with *wnt1* and *wnt10b* could counteract the effect of deleting *wnt*1 by promoting neurogenesis.

My data thus favours a model in which Wnt1 signals to adjacent cells to repress boundary fate by upregulating proneural and delta genes. Notch signalling is reinforced at boundaries by Delta in neighbouring cells, and maintains *wnt1* expression in boundary cells (Cheng et al., 2004). This mechanism links the patterning of neurogenesis to the maintenance of a sharp signalling centre.

Differences between the zebrafish hindbrain and the Drosophila *wing imaginal disc.*

Although this model appears remarkably similar to that regulating wing margin development in *Drosophila*, there are differences that may reflect distinct features of hindbrain development. First, the expression of *fringe* homologues and Notch ligands that establishes the stripe of Notch activation at the boundary is different, which presumably reflects the larger number of *fringe* genes in zebrafish whose function may have diverged (Dale et al., 2003). For instance, *lunatic fringe* is expressed in even-numbered segments in the zebrafish hindbrain (Prince et al., 2001), in a pattern which is closer to the expression of *fringe* in the dorsal compartment of the fly wing disc. Another difference is that Wnt signalling appears to regulate *asha* and *ashb*, the homologues of *achaete* and *scute*, as well as the *atonal* homologue *ngn1* in the zebrafish, while *atonal* is not

expressed in the wing disc. This reflects the greater diversity of neuronal types in the hindbrain.

Another point of difference is the regulation of *delta* expression by Wnt signalling. In the wing disc, *delta* and *serrate* are upregulated adjacent to the whole length of the boundary, while proneural genes are only expressed in the anterior compartment, indicating direct regulation by Wingless of these genes, independently of proneural function (which has been demonstrated in the case of *serrate*, Yan et al., 2004). In neural cells in vertebrates, however, proneural genes are necessary and sufficient for delta gene expression (Casarosa et al., 1999; Fode et al., 1998; Hans and Campos-Ortega, 2002; Ma et al., 1998), therefore I have presumed that Wnt regulates *deltaA*, *deltaB*, and *deltaD* indirectly, by regulating proneural gene expression. This assumption is difficult to verify in this context. There does, however, remain the possibility of a direct input of Wnt signalling into delta gene expression in the hindbrain, as the Wnt pathway has been recently found to directly regulate *dll1* expression in the pre-somitic mesoderm of mouse embryos (Galceran et al., 2004; Hofmann et al., 2004).

Another significant difference between the two systems is that, while Notch signalling is sufficient to instruct cells to adopt a boundary fate in the wing disc (de Celis and Bray, 1997; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; Micchelli et al., 1997; Rulifson and Blair, 1995), it does not have this role in zebrafish (Cheng et al., 2004). An implication of this is that the mechanisms by which repression of boundary fate by proneural and/or delta genes may be different from the mechanisms in the wing disc, where Delta cell autonomously blocks Notch activation. Current evidence suggests that blocking

Notch activity is not sufficient to abolish boundary formation in the hindbrain (Cheng et al., 2004). Consequently, the primary mechanism by which proneural and/or delta genes suppress boundary fate may involve another, as yet undetermined factor (factor "X" in Fig.21B). Indeed, if Notch activation induced boundaries, most of the hindbrain would consist of boundary cells, as most cells are likely to experience Notch signalling during normal development as a consequence of lateral inhibition. Boundaries appear to be defined by the absence of proneural gene expression in the zebrafish, and this may provide clues as to the genes that Wnt1 indirectly represses to suppress ectopic boundary formation. The experiments described in this work do not address the question of whether repression of boundary markers is carried out by proneural genes or by high levels of Delta protein, and this will need addressing in future work.

Contribution of different Wnt sources.

Another significant difference between the *Drosophila* wing disc and the zebrafish hindbrain is that, in the hindbrain Wnt1 is produced not only by boundary cells, but also by the roof plate. *wnt1* morpholino knockdowns block translation of both roof plate and boundary transcripts, and do not allow to assess whether boundary Wnt1 is important. However, in rfngMO embryos, expression of *wnt1* is lost at boundaries, but not in the roof plate (Cheng et al., 2004), and a phenotype similar to wnt1MO embryos of boundary marker expansion and reduction in neurogenesis is observed. Although it remains possible that knockdown of *rfng* affects other genes that could mediate a similar function, the simplest explanation is that the expansion of boundary markers observed following *rfng* knockdown is due to decreased *wnt1* expression at boundaries.

Nevertheless, this does not imply that the roof plate Wnt1 does not participate in this, as it would be difficult to conceive that the same protein derived from different cells would have different roles. Rather, the most likely hypothesis is that boundary-derived Wnt1 is required to elevate total levels of extracellular Wnt1 in the hindbrain above a certain threshold, which must be reached to block boundary marker expression.

A further complication is that at least five Wnts are expressed in zebrafish hindbrain boundaries : *wnt1*, *wnt8b* (this study), *wnt3a*, *wnt10b* (Riley et al., 2004) and *wnt4a* (referred to as *wnt4* in Blader et al., 1996). This could be one of the reasons why knockdown of *tcf3b* has a stronger phenotype than knockdown of *wnt1*. However, considering that there are four other Wnts at boundaries, the wnt1MO phenotype is surprisingly strong. One explanation could be that Wnt1 regulates expression of the other Wnt genes, and this is indeed the case for *wnt8b*, which is no longer detected in rhombomere boundaries following *wnt1* knockdown. I have not yet tested this possibility for the three remaining Wnt genes. Nevertheless, even in the case of *wnt8b*, expression is not abolished in wnt1MO embryos, and remains in the roof plate. As roof plate Wnt1 is able to contribute to repression of boundary markers and promotion of neurogenesis, this explanation cannot account for the strength of phenotype after wnt1MO injection. An alternative is that all these Wnts are necessary to achieve a threshold concentration throughout the hindbrain, and that disrupting Wnt1 is enough to lower the concentration below that threshold. Arguing against this is the fact that in embryos injected with morpholinos against both *wnt8b* and *wnt3a*, *deltaA* expression is not significantly reduced (Riley et al., 2004). Although this could imply that Wnt1 is the major contributor to Wnt protein levels, this is

unlikely. Instead, this finding suggests that different Wnts may have different activities and regulate different aspects of boundary and hindbrain development. Many studies have found that "canonical" Wnts have different roles, either by eliciting a response from one cell type but not another, or by eliciting different responses from the same cell type (for instance, different midbrain neurons respond to Wnt3a than to Wnt1 and Wnt5a, and within the same group of neurons, Wnt1 affects primarily proliferation, while Wnt5a has a direct effect on fate, Castelo-Branco et al., 2003; and in the spinal cord, only Wnt1 and Wnt3a, but not Wnt3,4,7a or 7b, cause ectopic proliferation, Megason and McMahon, 2002).

Thus, the most likely model for the action of Wnts in the hindbrain is that Wnt1 is required at threshold levels to achieve repression of boundary fate and promote neurogenesis, while other Wnts may contribute to this threshold but some may have other roles.

Evolutionary conservation of a regulatory gene network.

That the Notch/Wnt/proneural pathway that has been identified in this work and in previous work on the *Drosophila* wing disc should be so similar suggests that it constitutes an evolutionarily conserved genetic module. Some aspects of this network appear to have been recruited in other contexts. For instance, ectopic Notch activation can lead to *wnt1* expression in other vertebrate tissues such as developing nails (Lin and Kopan, 2003). Similarly, loss of Notch2 function in the midbrain leads to disorganised and reduced *wnt1* expression in the roof plate, and ubiquitous expression of *notch3* may account for remaining expression (Kadokawa and Marunouchi, 2002).

Other regulatory interactions in the pathway are also found in many systems. The regulation of proneural genes by Wnt signalling has been described in *Drosophila* Malpighian tubules, where, similarly to the results of this study, *wingless* is not required at early stages of *achaete* gene expression. However *achaete* gene expression is lost in a *wingless* mutant at later stages (Wan et al., 2000). In the vertebrate intestine, stem cells can differentiate into either absorptive or several types of secretory cells, the latter requiring the expression of a proneural-related bHLH gene, *Math1* (reviewed in Sancho et al., 2003). Inhibition of Wnt signalling leads to a loss of proliferating cells and also of all the secretory lineages, implicating Wnt signalling in the regulation of *Math1* (Pinto et al., 2003). In neural tissue, recent work has found that β-Catenin/TCF complexes can directly regulate *Neurogenin1* expression by binding to its promoter (Hirabayashi et al., 2004; Israsena et al., 2004).

A further situation where many similarities with the Notch/Wnt pathway are observed is in patterning of the limb bud. A structure called the apical ectodermal ridge (AER) is responsible for outgrowth, similar to the margin of the fly wing. Interestingly, *radical fringe* has been implicated in the formation of the AER (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). Mutation of the Notch ligand *jagged2* results in expansion of the AER (Jiang et al., 1998), as does reducing repression of the Wnt pathway in the doubleridge mutant mouse, where expression of the Wnt antagonist *dkk1* is reduced (Adamska et al., 2003; MacDonald et al., 2004). This implies a relationship between Notch and Wnt signals, although in this instance, they appear to have opposing effects. Indeed, several studies have shown that Wnt/β-Catenin signalling can induce AER formation (Barrow et al., 2003; Kawakami et al., 2001; Kengaku et al., 1998;

Soshnikova et al., 2003). A potential explanation is that in the limb buds, *cux1*, a homologue of the *Drosophila* gene *cut*, is expressed adjacent to the AER, and is possibly induced non cell-autonomously by β-Catenin (Tavares et al., 2000). Cux1 appears to have opposing effects to Wnts, and represses AER markers, which is similar to its role in the wing disc, where it renders cells unable to respond to Wingless (Micchelli et al., 1997). Thus, boundary restriction is achieved using homologous genes, albeit expressed with different spatial relationships. Many details of this pathway are still to be elucidated, but insights from the role of genes and their interactions at the *Drosophila* wing margin may help to advance understanding of limb development. Finally, an intriguing parallel is observed in the inner ear of chick and mouse embryos, where overactivation of the Wnt pathway (Stevens et al., 2003) or of the Notch pathway (Daudet and Lewis, 2005) both cause the appearance of ectopic sensory patches, suggesting that a similar regulatory gene network involving Wnt, Notch and sensory genes may also regulate hair cell formation in the inner ear.

It is striking, therefore, that the spatial relationships between homologous genes in the zebrafish hindbrain and the wing margin are so similar, and that the similarities cover the whole pathway, unlike the other examples presented. Many of the differences between the genetic networks described in the zebrafish hindbrain and the *Drosophila* wing can be attributed to a higher complexity of the tissue in the hindbrain, as well as a higher genomic complexity. Nevertheless, the parallels between the regulatory hierarchies are remarkable, since they cannot be due to the two structures being homologous. It seems likely that the similarities are due to independent recruitment of a regulatory network of genes

that has a key role in organising boundary cells by linking the patterning of adjacent tissue to the prevention of the spread of the signalling source.

Discussion and perspectives.

Regulation of neurogenesis.

Mechanisms of neural patterning in the zebrafish hindbrain.

Analysis of markers for neuronal differentiation and for individual neural cell types, and the finding that Wnt signalling is required for neurogenesis after 18h in the hindbrain have provided new insights into the mechanisms organising the anterior-posterior neural pattern in each rhombomere. Several lines of evidence suggest that this patterning involves two processes : the restriction of neuronal differentiation to particular areas along the anterior-posterior axis, and the subsequent movement of differentiated cells to their final location. The latter process suggests that there may be an affinity gradient regulating cell position along the anterior-posterior axis of each rhombomere and maintaining each cell type at its appropriate location. Both mechanisms are likely to involve boundaries as the source of signals regulating positional information, implying that gradient landscapes along the anterior posterior axis of each rhombomere have a low point at the centre of each rhombomere. My work has not addressed the question of how final cell position is regulated, but has identified a mechanism regulating neuronal differentiation in the hindbrain.

How is the localisation of differentiation determined ?

The spatial regulation of neurogenesis requires that proneural gene expression is localised to the appropriate areas. This is particularly clear at early somitogenesis stages, when *ngn1* expression is restricted to a few cell clusters in the hindbrain that will give rise to primary neurons (Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998). At later stages, this requirement for spatial regulation of proneural genes is less evident, as low-level proneural gene expression is detected the hindbrain, except at boundaries. Nevertheless, as *wnt1* knockdown leads to decreased proneural gene expression, it is clear that it is important to positively regulate their expression in order for neurogenesis to occur.

However, expression of proneural genes is not enough to determine where differentiation occurs. In *Drosophila*, during specification both of neuroblasts and of macrochaete, there is evidence that, after proneural clusters are established, extrinsic signals bias the lateral inhibition process so that the same cell within a cluster is always specified as the macrochaete precursor or neuroblast (reviewed in Simpson, 1997). Similarly, in the zebrafish hindbrain, the location of differentiating neuroblasts is stereotyped, but at 18h and 24h, proneural and Delta gene expression is broader than this area (Fig.7). Work carried out in the Wilkinson lab has suggested that the early expression of Delta genes has a role in activation of the Notch pathway at hindbrain boundaries (Cheng et al., 2004). High and low levels of *deltaA* and *deltaD* expression are detectable at 24h (Fig.7). The high levels correspond to cells also expressing *deltaB* and $p27^{xic1}$-*a*, indicating that these cells are in the process of

differentiating. Low-level expression presumably indicates cells in a proneural state, which can initiate a programme of neuronal differentiation, but are maintained as progenitors by lateral inhibition.

Therefore, spatial patterning of neuronal differentiation requires two steps : first that clusters of cells are in a state of competency to differentiate, and second, that a bias determines which cells within these clusters do differentiate.

Establishment of proneural clusters.

Recently, genes of the Iroquois family have emerged as candidates for patterning primary neurogenesis. Iroquois complex members in *Drosophila* are among the prepatterning genes involved in determining where proneural genes can be expressed (Cavodeassi et al., 2001; Gomez-Skarmeta et al., 2003; Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996). In the vertebrate hindbrain, Iroquois genes are expressed at very early stages of segmentation in domains with distinct anterior and posterior boundaries, and regulate *ngn1* expression in the zebrafish as well as in *Xenopus* (Calle-Mustienes Ede et al., 2002; Glavic et al., 2002; Gomez-Skarmeta et al., 1998; Itoh et al., 2002; Lecaudey et al., 2004). It seems likely therefore, that Iroquois genes, probably in combination with other factors, contribute to the pattern of reticulospinal neuron development. Moreover, comparisons with *Drosophila* neurogenesis seem more warranted for the early pattern of neurogenesis in the hindbrain than for later vertebrate neurogenesis. Whereas most neurogenesis produces many cells of the same type from single progenitor domains, early neurogenesis in the zebrafish hindbrain gives rise to a defined set of individually recognisable cells at particular, reproducible locations. This is similar to the situation in the fly, where

proneural clusters have a stereotypical shape, and the cell that is selected to become neuronal is found at a constant position within the cluster, such that the neuronal cells are found at the same location in the tissue (Cubas et al., 1991; reviewed in Gomez-Skarmeta et al., 2003; Skeath and Carroll, 1991). However, this does not exclude a potential role for Iroquois genes in the regulation of proneural competency at later stages, as many of these genes are expressed in the hindbrain at 24h (Feijoo et al., 2004; Tan et al., 1999; Wang et al., 2001).

After 18h of development, expression of proneural genes requires Wnt1. Around the same time, their expression patterns change from segmental patterns to stripes of high level expression adjacent to rhombomere boundaries, and low levels throughout rhombomere centres. This suggests that different mechanisms regulate proneural gene expression before and after this point. It is possible that Wnt1 directly regulates proneural gene expression, as TCF/β-Catenin complexes bind to the *neurogenin1* promoter in mouse (Hirabayashi et al., 2004; Israsena et al., 2004). However, as discussed above, it seems likely that Wnt signalling acts in a permissive manner for proneural gene expression. Work in *Drosophila* has found that Wingless signalling acts permissively to establish proneural clusters during microchaete specification (Ramain et al., 2001). Wingless functions to inhibit a Suppressor of Hairless-independent function of Notch, in which Notch signals via Deltex to block the establishment of proneural clusters. This regulation takes place prior to the Suppressor of Hairless-dependent lateral inhibition that occurs subsequently. It is thus likely that many different mechanisms are responsible for the regulation of proneural competency, including repression (at boundaries, for instance), direct activation, and relief of repression.

Biasing differentiation within proneural clusters.

The second step in patterning the location of differentiation is to bias the lateral inhibition process, such that the location of cells that are selected to differentiate is fixed and stereotyped. Interestingly, in *Drosophila*, Wingless can signal to initiate this bias (Simpson, 1997). Bias can be obtained through elevated levels either of proneural or Delta genes, and, as discussed previously, Delta gene promoters in both zebrafish and mouse contain TCF binding sites (Galceran et al., 2004; Hofmann et al., 2004). Thus the bias in the hindbrain, giving rise to differentiation adjacent to boundaries, could be achieved in this way by Wnt signalling. However, Wnt1 is unlikely to be responsible, due to its dorsally restricted expression. Other Wnts, most notably Wnt4a (ZfWnt4 in Blader et al., 1996), are expressed more extensively in boundaries, and may have a role in this type of patterning.

It is interesting to note that at 18h and at 24h, proneural genes are expressed throughout the ventricular zone in non-boundary regions, but that differentiation occurs mainly in the centre of rhombomeres at 18h and adjacent to boundaries at 24h. This implies that signals that regulate differentiation in the proneural equivalence groups vary between these time points. There is evidence to support this idea : for instance, it is from 18h that *wnt8b* expression begins to be upregulated in boundaries. Boundary cells may thus act to promote neuronal differentiation at close range after 18h. Conversely, it is possible that neuronal differentiation is inhibited by local signalling events in rhombomere centres. Dynamic gene expression in non-boundary regions is also observed between 18h and 24h, such as the ETS-related *erm* transcription factor, which is upregulated

in rhombomere centres (Munchberg et al., 1999), although no evidence links this gene to the regulation of neurogenesis.

Boundaries and neurogenesis.

An interesting aspect of neurogenesis in the hindbrain is that boundaries do not express proneural genes, suggesting that they do not generate any neurons. It is plausible that the radial glia located adjacent to boundaries are boundary-derived, but another possibility is that when boundary cells divide, one of the daughter cells finds itself in the adjacent, neurogenic, area, and produces neurons. An implication of this second hypothesis is that boundary fate is not inherited, but instead requires a constant, localised input for its maintenance.

The mutually exclusive spatial relationship between boundary cells and proneural gene expression suggests that these are two separate identities, that cross-repress each other. Indeed, when proneural genes are knocked down, boundary markers expand to fill entire rhombomeres. Conversely, work in the Wilkinson lab has shown that overexpression of *ngn1* leads to repression of boundary markers such as *rfng* (Y.-C. Cheng, unpublished observations). The mechanism by which proneural genes repress boundary fate is not known. One possibility is that Delta gene expression, which depends on proneural genes, acts in a dominant negative manner on Notch activation, as occurs at the wing margin during *Drosophila* development (de Celis and Bray, 1997; Micchelli et al., 1997). This model requires that Notch activation be a necessary condition for boundary cell fate specification. An alternative is that proneural genes act,

directly or indirectly, at the level of transcription to repress *rfng* and other boundary-specific genes. Equally, a mechanism must be present to prevent boundary cells from expressing proneural genes, and render them insensitive to Wnt signalling. In the case of the wing margin, a single transcription factor, Cut, represses the Wingless targets *delta* and *serrate*, and thus ensures that the margin cells remain distinct from their neurogenic neighbours (de Celis and Bray, 1997; Micchelli et al., 1997). Such a transcription factor that acts as a selector gene for boundary fate remains to be found in vertebrates.

Conservation and potential significance of anterior-posterior patterning within rhombomeres.

Clues as to the functional importance of the patterning of neurogenesis along the anterior-posterior axis within rhombomeres may be gleaned from examining conservation between species. Medaka fish (*Oryzias Latipes*) have stripes of expression of *deltaA* (Candal et al., 2004), and of a cyclin-dependent kinase inhibitor (Nguyen et al., 2001) within rhombomeres, reminiscent of those observed with zebrafish $p27^{xic1}$-*a*. This suggests that the patterns, and probably the mechanisms, of hindbrain neuronal differentiation are common between teleosts. There are few descriptions of neurogenesis in the hindbrain of *Xenopus* at stages equivalent to those in this study, but *Xash1*, the homologue of *ash* genes, is expressed throughout the hindbrain, except in stripes that probably correspond to rhombomere boundaries (Ferreiro et al., 1993). However, no stripes of elevated expression adjacent to boundaries have been described, which

suggests that late neurogenesis is not spatially restricted, except in so far as boundary cells do not express proneural genes. In the case of early neurogenesis in *Xenopus*, reticulospinal neurons are among the earliest neurons to develop in the hindbrain, but individual cells do not appear to have particular locations within rhombomeres. Instead, reticulospinal neurons are present in continuous columns in the hindbrain, with the exception of the Mauthner neuron, which is unique and restricted to r4 (Hartenstein, 1993; Nordlander et al., 1985; Sanchez-Camacho et al., 2002; van Mier and ten Donkelaar, 1984). In mouse, neuronal markers and proneural genes are expressed in longitudinal columns, which are mostly continuous along the whole hindbrain (often with the exception of r4, which is dorsally shifted, Davenne et al., 1999; Gavalas et al., 2003). The Notch ligand homologues *delta* and *serrate/jagged* are also expressed in longitudinal columns both in mouse and chick (Kusumi et al., 2001; Myat et al., 1996). Indeed, while there may be differences in neuronal phenotypes between rhombomeres, within rhombomeres neurons are arranged in continuous columns (Clarke and Lumsden, 1993). That there are differences between fish and other vertebrates in neuronal organisation within segments implies that different mechanisms may pattern neurogenesis. The functional importance of having anterior-posterior organisation of neuronal types is not clear. Presumably, it must correspond to some organisation of neural circuits in aquatic organisms, but of circuits that are not required for tadpoles. It is intriguing that, although tadpoles swim and have escape reactions involving reticulospinal neurons, similar to zebrafish, the circuits regulating these responses do not appear to require clustering of reticulospinal neurons, as occurs in the zebrafish hindbrain (van Mier and ten Donkelaar, 1989). Finally, in my experiments, two day-old

zebrafish larvae in which *wnt1* has been knocked down appear to have normal movements, although detailed studies have not been carried out. These embryos lack most neurons born after 18h. Therefore, it may be that the organisation of late-born neurons along the anterior-posterior axis of each rhombomere is important for functions in adult fish.

However, unlike the pattern of neurogenesis, which seems to be restricted to teleosts, the presence of radial glia at rhombomere boundaries is a feature conserved throughout evolution. It has been shown that vimentin, a radial glial marker (Dahl et al., 1981), is expressed at high levels in hindbrain boundaries in chick (Heyman et al., 1995), in *Xenopus Laevis* (Yoshida, 2001; Yoshida and Colman, 2000), and in alligator embryos (Pritz, 1999). The significance of this seems to differ between species ; whereas in chick increased vimentin is associated with axon outgrowth at boundaries (Heyman et al., 1995; Heyman et al., 1993; Lumsden and Keynes, 1989), no increase in axon density has been described at boundaries in *Xenopus*. *Xenopus* boundary radial glia are the only proliferating cells in the hindbrain at late stages (Katbamna et al., 2004). In zebrafish, glia appear to fulfil a combination of both these roles. Early axons populate the centre of rhombomeres, but later commissural axons follow the glial fibres (Trevarrow et al., 1990). However, the glia also proliferate, and by 48h are the only non-neuronal cells in the hindbrain (Lyons et al., 2003), implicating these cells not only in structural organisation of neuronal connectivity, but most likely also in the process of neurogenesis. Indeed, in the cerebellum of the adult teleost *Apteronotus* leptorhynchus, radial glia are neuronal progenitors (Zupanc and Clint, 2003). An important aspect of the role of the radial glia in the

zebrafish hindbrain may be that they are involved in guiding the migration of cell bodies of differentiating neurons out of the ventricular zone. This is similar to the role that radial glia are thought to fulfil in the mammalian cortex (Rakic, 1978), where newly born neurons use radial glial processes as a substrate for migration into the cortical plate. The radial glial curtain adjacent to boundaries could provide a good substrate for migration of neurons into the mantle zone, as neurogenesis occurs adjacent to boundaries. Thus, differentiating neurons are immediately adjacent to radial processes that they can migrate along, and once they reach the mantle zone, they can migrate along the anterior-posterior axis or stay adjacent to boundaries, as in the case of *dbx1*-positive neurons. In chick, however, as there is no evidence of localised neuronal differentiation adjacent to boundaries, radial glia probably fulfil other roles at boundaries, such as stabilising boundaries, or guiding axons. Therefore, although the presence of glia at rhombomere boundaries is a conserved feature of vertebrate evolution, they have been recruited for different uses in different organisms.

The second aspect of anterior-posterior patterning within rhombomeres that appears to be conserved is the relationship between boundaries and proneural genes. In *Xenopus*, for instance, *Xash1* is not expressed at boundaries (Ferreiro et al., 1993). In chick embryos, the expression patterns of proneural genes have not been described with respect to boundary expression in the hindbrain, but *id1* whose product antagonises proneural gene function is expressed at high levels in boundaries (Kee and Bronner-Fraser, 2001). Thus, it is likely that boundary cells generate fewer neurons than non-boundary cells in many vertebrates, and this may be important to maintain a stable population of boundary cells.

New insights into the roles and formation of rhombomere boundaries.

Previously, little had been known about the role of rhombomere boundaries, and it was suggested that their primary role was to stabilise interfaces and thus inhibit cell mixing between segments (Guthrie et al., 1991). Recently published work supports this idea by showing that boundary cells have specific affinity properties, which are modulated by Notch activation (Cheng et al., 2004). Here, I show that zebrafish rhombomere boundaries are signalling centres that regulate neurogenesis in the hindbrain. Moreover, I have found that following decreased Wnt signalling, boundary cells can be induced away from segmental interfaces, implying that the mechanism of boundary formation is more complex than previously thought.

Notch and Wnt1 link boundary maintenance to the regulation of neurogenesis.

Taken together with work we have previously published (Cheng et al., 2004), my results reveal a regulatory loop between boundary and non-boundary cells that stabilises the identity of each population via bidirectional lateral inhibition. Sustained Notch activation in boundary cells, modulated by Rfng, prevents their premature differentiation and regulates their affinity properties,

thereby maintaining their segregation to segment interfaces. Notch activation also upregulates *wnt1* expression in hindbrain boundary cells. Notch activation is maintained by expression of delta genes in adjacent cells (Cheng et al., 2004). I have found that Wnt1, expressed by boundary cells, is required for the expression of delta and proneural genes in non-boundary cells, which enables neuronal differentiation and laterally inhibits the spread of boundary marker expression. Expression of *wnt1* in the roof plate is independent of Rfng function, and contributes to the promotion of neurogenesis, but is not sufficient either for the normal level of neurogenesis, or to block the spreading of boundaries. Thus, tissue patterning and maintenance and restriction of boundaries are linked in a bidirectional feedback loop involving Notch, Wnt1 and proneural and delta genes.

In order for localised signals to pattern the adjacent tissue, it is necessary that the amount and location of signal is precisely regulated. Thus the interface at which the signalling source is induced must remain sharp and the location and number of signalling cells must be tightly controlled (Dahmann and Basler, 1999). The molecular pathway that occurs in hindbrain accomplishes this : Notch regulates the segregation of boundaries, while inhibiting their differentiation, and Wnt1 ensures adjacent cells do not become boundary cells, whilst reinforcing Notch activation in boundaries via proneural and delta gene expression. These processes ensure that signalling from hindbrain boundaries is maintained at restricted locations and at relatively constant amounts.

How do boundaries form ?

Whilst the model presented above explains how boundaries are maintained, it does not address the question of how they are induced in the first place. It is known that boundaries are induced when odd- and even-numbered rhombomeres are juxtaposed, but not when segments of the same parity are made to interact (Guthrie and Lumsden, 1991). Both Notch and Eph/ephrin pathways are candidates for inducing boundaries as they display differences between odd- and even-numbered segments in different species from mouse to zebrafish, such that activation of the pathway would occur only at the interface between segments of different parity.

The genes involved in the regulatory loop revealed in this work (Fig.21B) do not affect initial boundary formation. Notch signalling appears to be neither required nor sufficient for the initial expression of boundary markers such as *foxb1.2* and *rfng* (Cheng et al., 2004). Similarly, boundary-restricted genes are not required for boundary formation, as knockdown of *wnt1*, *pax6*, *rfng* or of *foxb1.2* does not lead to loss of boundary markers (this work, preliminary data not presented and *foxb1.2* knockdown carried out by Yi-Chuan Cheng). Moreover, boundaries are visible by morphological analysis before any currently known markers are expressed there (all boundaries can be detected as early as the 8 somite stage (12.5-13h), Moens et al., 1998). It is possible, however, that boundary cell morphology is simply due to cells at segment interfaces being exposed to Eph/ephrin signalling, for instance, and change their shape due to repulsive interactions. Thus, morphological boundaries may be formed before boundaries are induced as a distinct cell type.

Notch signalling.

Although boundaries are still present in *mind bomb* mutants that have a strong deficiency for Notch signalling, there is residual Notch activity in these mutants (Cheng et al., 2004; Itoh et al., 2003), so that it cannot be excluded that low levels of Notch signalling may be required, if not instructive for boundary formation. Two observations support a role for Notch in the early development of boundaries ; one is that activating Notch signalling drives cells to sort to segment interfaces as early as 13.5h of development (Cheng et al., 2004). The second is that zebrafish *lunatic fringe* (*lfng*) is expressed in even-numbered rhombomeres from the three somite stage (Prince et al., 2001). Lfng could be involved in modulating Notch activity at the interfaces of its expression domains, in a similar manner to the role of its homologue, *fringe*, in establishing the dorso-ventral boundary of the wing disc in *Drosophila* (Panin et al., 1997). Moreover, the observation that *lfng* is also expressed in alternating segments in mouse suggests that this role may be conserved in development (Johnston et al., 1997). Since mouse *lfng* is expressed in odd-numbered rhombomeres (rather than even-numbered in zebrafish) indicates that the significance of its expression may be to define interfaces of differential Notch sensitivity to its ligands, rather than roles within each segment. Nevertheless, Notch signalling alone is not sufficient to induce ectopic boundaries, and must therefore cooperate with other signals for boundary formation.

Eph/ephrin signalling.

Obvious candidates for mediating interactions specifically at rhombomere interfaces are the ephrin transmembrane family of proteins and the ephrin-

interacting receptor tyrosine kinases, Ephs (reviewed in Cooke and Moens, 2002; Xu et al., 2000). The alternating expression of Eph receptors and ephrins in odd- and even-numbered rhombomeres leads to bidirectional signalling at segment interfaces (Cooke et al., 2001; Mellitzer et al., 1999; Xu et al., 1999). Furthermore, there is increasing evidence that both Ephs and ephrins can regulate gene transcription, in addition to their known roles in regulating cytoskeletal assembly/deassembly (Lai et al., 2004; Li et al., 2001; Takasu et al., 2002). It has also been shown that disrupting Eph/ephrin signalling leads to cell intermingling and absence of boundary markers in the zebrafish hindbrain (Xu et al., 1995). Similarly, loss of boundaries upon retinoic acid treatment of the chick hindbrain is closely correlated to loss of *ephA4* expression (Nittenberg et al., 1997), although many other cellular processes may be affected by this treatment. However, a fundamental problem for analysing roles of Eph/ephrin signalling in boundary formation is that disrupting this signalling leads to cell mixing between segments (Xu et al., 1995). Thus it is difficult to distinguish whether the absence of boundaries observed is due to a direct input of Eph/ephrin signalling into boundary formation, or whether it reflects that boundary induction requires a stable cell population at interfaces, which is destabilised when Eph/ephrin signalling is impaired.

Moreover, there are many difficulties to overcome when examining the role of Eph/ephrin signalling in boundary formation. The large number of Eph receptors and ephrin ligands expressed in the hindbrain mean that no phenotypes are observed in mouse knockouts, probably due to functional redundancy (reviewed in Cooke and Moens, 2002). Moreover, many receptors and ligands are expressed in overlapping, not complementary patterns, which may affect the

intensity and the outcome of signalling, as may interactions with other signalling pathways (reviewed in Poliakov et al., 2004). Thus, no signalling system has as yet been directly implicated in boundary cell induction.

Lessons from rhombomere 4.

The observation that different cell responses are observed in different experiments from cells in r4 poses many questions, but may also help in addressing fundamental questions regarding boundary formation and regulation. First, the presence of a Notch-regulated cell affinity difference in the centre of r4 is intriguing (Cheng et al., 2004). Cells expressing dominant-active Suppressor of Hairless sort to the centre of r4, as well as boundaries, while cells expressing dominant-negative Suppressor of Hairless sort away from the centre of r4. This may reflect the fact that r4 is the first rhombomere to differentiate (Maves et al., 2002). However, this explanation is not fully satisfactory, as the kinetics of cell sorting in r4 are not consistent with the appearance of gaps in delta gene expression in the centre of r4. An alternative hypothesis is that Notch signalling regulates differential affinity within segments in order to maintain the anterior-posterior organisation of neuronal and glial cell types within each rhombomere, or simply to limit cell movement before this organisation is established. Instead of the model stated above in which affinity states are graded across the anterior-posterior axis of rhombomeres, this finding may imply that affinity differences form discrete stripes that partition rhombomeres. That only r4 has this regulation implies that its integrity is important for hindbrain development, and may reflect

the fact that r4 is a signalling centre that regulates the development of r5 and r6 (Maves et al., 2002). A further implication of this pattern of Notch-regulated affinity is that Notch activity may not be regulated solely by its ligands and known modulators (such as fringe genes), as no difference is seen in the expression of these genes between r4 and other rhombomeres.

Further insights into boundary formation can also be gleaned from the pattern of expansion of boundary markers in Wnt pathway knockdowns. As *rfng* expression is not expanded into r4, it can be inferred that boundary formation is inhibited in r4. This raises the question of whether the generation of boundaries in normal embryos is symmetrical, that is to say whether odd- and even-numbered rhombomeres can contribute equally to boundary formation. Drawing again from comparisons with the wing imaginal disc of *Drosophila*, two radically different models of boundary formation can be envisaged (reviewed in Dahmann and Basler, 1999). In the first case, one segment/compartment signals to cells at the border of the other to become boundaries, as in the case of *hh* inducing boundary cells at the anterior-posterior boundary of the wing disc. The alternative model is that a bidirectional signal at the interface causes one cell from each side to adopt a boundary fate, as occurs at the dorso-ventral boundary of the wing disc. It may be the case that a combination of these mechanisms occurs as all rhombomeres appear to be able to generate boundary cells except r4, such that bidirectional boundary formation may occur at the r2/r3 and r5/r6 boundaries, while it may be unidirectional at the r3/r4 and r4/r5 boundaries. Alternatively, it is possible that r4 normally generates boundary cells, but that

boundary spreading in r4 is inhibited by a mechanism independent of the Wnt pathway.

Similarly, neurogenesis, as detected by continued expression of proneural genes, is less affected by loss of Wnt1 in r4 than in other rhombomeres. Interestingly, at early stages *wnt1* is not expressed in the roof plate of r4, nor is *wnt8b*. Thus, in a normal situation, r4 is exposed to less Wnt than other rhombomeres and presumably uses other mechanisms to ensure that neurogenesis does occur. The lower dependence of r4 on Wnt signalling implies that it must be important to pattern this particular rhombomere correctly.

Nevertheless, proneural gene expression is severely reduced in r4 in Wnt1MO embryos, and *gfap* expression is strongly increased, as in other rhombomeres. This observation suggests that boundary cells are not simply neural cells that do not express proneural genes. Moreover, in embryos when *asha*, *ashb* and *ngn1* functions have been impaired, no expression of *rfng* is seen in r4. The implication of these observations is that, while proneural gene expression may be incompatible with boundary fate, the latter is not a default identity in the absence of proneural gene expression.

It is possible that the distinct properties of r4 in boundary formation and neurogenesis reflect to its role as a signalling centre responsible for specifying the posterior hindbrain (Maves et al., 2002). This organising function of r4 may require that it is more stable than other areas. It is not known what mediates this stability. *fgf3* and *fgf8* are expressed in r4 and mediate the patterning properties of r4 on adjacent segments (Maves et al., 2002). It is possible that the high levels of FGF signalling experienced by r4 modulate its response to other signals,

including boundary-inducing signals. However, expression of two FGF targets, *erm* and *pea3*, is uniform in the hindbrain at early somitogenesis stages (Maves et al., 2002; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). This suggests that it is not FGF signals that confer the distinct properties of r4, and better candidates are genes that are specific to r4, such as *hoxb1a* for instance.

What are the roles of rhombomere boundaries ?

Few studies have addressed the role of hindbrain boundaries, but several hypotheses have been put forward. In addition to possibly inhibiting cell mixing between adjacent rhombomeres, or stabilising interfaces (Guthrie et al., 1991; Lumsden, 1990), it has been suggested that they may block the spread of signals and restrict domains of gene expression due to the absence of gap junctions (Martinez et al., 1992), or act as axon scaffolds (Heyman et al., 1995; Heyman et al., 1993; Lumsden and Keynes, 1989). A further possibility is that they specify neuronal and glial patterns within segments.

Inhibition of cell mixing across interfaces.

A suggested role of boundaries is that they could restrict cell mixing between segments. This idea has been reinforced by the recent observation that the affinity of boundary cells is different to that of non-boundary cells (Cheng et al., 2004), suggesting a model in which two different cell affinity mechanisms regulate affinity differences between adjacent segments (the Eph/ephrin system) and between boundary and non-boundary cells (driven by Notch activation at

boundaries). This would lead to greater stability across an interface than if a single mechanism operated. However, it appears that disruption of Eph/ephrin signalling alone is sufficient to cause intermingling between rhombomeres (Xu et al., 1995). This may reflect that this pathway is important in restricting movement at stages before the Notch pathway has stabilised interfaces. Moreover, cell labelling experiments show that cell mixing is inhibited between cells from adjacent segments, but not between boundary and non-boundary cells (Fraser et al., 1990; discussed by Lumsden, 1990). Similarly, when boundaries are ablated or not present due to retinoic acid treatment, no significant intermingling is observed across interfaces (Guthrie and Lumsden, 1991; Nittenberg et al., 1997). Thus, it seems likely that the restriction of mixing between segments is due to interactions at segment interfaces, and not to boundary cells. However, there is some evidence for a role of Notch signalling in maintaining sharp interfaces between adjacent rhombomeres. In the *mind bomb* mutant, for instance, where Notch signalling is strongly impaired, the borders of the EphA4 domains are not as sharp and straight as in wild type embryos (Cheng et al., 2004). However, in knockdowns of *rfng*, which may promote Notch activation in boundaries, *epha4* and *krox20* expression are normal (Yi-Chuan Cheng, unpublished observations).

Although boundary cells are not responsible for the inhibition of mixing across segment interfaces, they have distinct affinity properties (Cheng et al., 2004), and may be less motile than non-boundary cells (Guthrie et al., 1991). It is possible that these properties are required for maintaining cell organisation within segments, rather than between segments. It would be interesting to see whether cell movement is increased following boundary ablation, or conversely,

whether boundary expansion leads to decreased movement, to test the possibility that boundary cells are less motile than non-boundary cells. Instead, if a model of graded affinity across segments is true, boundary ablation and boundary expansion should lead to similar increases in movement, as in both cases, the effect would be to level affinity across all cells in a segment.

Restriction of gene expression domains.

Another possibility is that boundaries are important to maintain the homogeneity of gene expression within segments. This idea derives from observations that boundary cells do not share cell-junctional communications with other cells and that they can inhibit the inductive capabilities of isthmic cells (Martinez et al., 1992; Martinez et al., 1995). In addition, it is known that, within each rhombomere, there are "community effects" such that if individual cells are in the wrong segment, they can switch identity to that of their new environment, but groups of cells can maintain their old identity (Pasini and Wilkinson, 2002; Schilling et al., 2001; Trainor and Krumlauf, 2000a; Wilkinson, 1995). Moreover, experiments showing that Krox20 can induce its own expression non-autonomously provide evidence for signals mediating community effects (Giudicelli et al., 2001). Thus, it can be conceived that boundaries limit the spread of the signals mediating these community effects, ensuring the isolation of each compartment. In this way, signals produced by one compartment to maintain that segment's identity and homogeneity do not affect neighbouring compartments. As the nature of these signals is not known, it is currently not possible to test this idea. However, it can be suggested that when most cells in the hindbrain adopt a boundary fate, the signals that maintain

segment identity cannot diffuse as far as they normally do, and in that way, it could be conceived that some cells may lose their identity. Nevertheless, when boundaries expand to fill most of the hindbrain, segment identity markers are not affected. This argues that the signals can be transmitted by or across boundary cells. Indeed, it may be that it is interfaces rather than boundary cells that restrict the spread of signals. This is consistent with the finding that Eph/ephrin signalling can restrict gap-junctional communication across interfaces (Mellitzer et al., 1999). In this model, boundary cell spreading would not affect communication within or between segments, an idea conforming to the results I have obtained.

Guiding axons.

Work in the chick has established that early axons populate hindbrain boundaries (Heyman et al., 1993; Lumsden and Keynes, 1989). A role in axon guidance is further suggested by the presence of specialised extracellular matrix at boundaries, as well as vimentin labelling, suggesting that at least some boundary cells are glia (Heyman et al., 1995; Lumsden and Keynes, 1989). Thus it may be that an important role of rhombomere boundaries is to act as a scaffold, to pattern and support axon growth. However, in zebrafish embryos, early axons develop in the centre of rhombomeres (labelled with anti-acetylated tubulin, for instance, Jiang et al., 1996), and although axons are later seen in boundary regions, they follow the curtain of radial glial processes which are adjacent to the boundaries (Trevarrow et al., 1990). Nevertheless, zebrafish hindbrain boundaries express *netrin1a*, which acts as either a chemoattractant or a chemorepellent for axonal growth cones (Lauderdale et al., 1997). Moreover, in

the zebrafish, I have shown that boundaries regulate the expression of glial markers, and the distribution of radial glial fibres, implying that they do have an input, even if indirect, into the organisation of axonal fibres and tracts. The observation that the spatial relationship between boundaries and axons is different between zebrafish and chick hindbrain suggests that this role is not the primary role of boundaries, and that different mechanisms have taken advantage of boundaries to pattern axon tracts in different species.

Boundaries as signalling centres.

A final possibility, which has been confirmed in this work, is that a major role of rhombomere boundaries is to function as signalling centres. It appears likely that the localisation of neuronal differentiation is patterned by boundaries, and that the final location of neurons could also be regulated and maintained by boundary-derived signals. I have shown that *wnt1* is expressed in boundary cells and regulates neurogenesis in rhombomere centres. It is unlikely that Wnt1 is a morphogen regulating cell type, or that it directly regulates the localisation of neuronal differentiation. Several other Wnts are also expressed in boundaries in the zebrafish (Blader et al., 1996; Riley et al., 2004), and it is possible that some Wnts may have a role in organising neurogenesis, rather than enabling it as in the case of Wnt1. Alternatively, other signals expressed at boundaries may regulate the anterior-posterior organisation of cell types within hindbrain segments.

In other species, it seems likely that rhombomere boundaries also secrete signals. In mouse, elevated levels of *wnt1* transcript are detected at boundaries (Echelard et al., 1994), although it is not clear how far the expression extends ventrally. In chick and mouse, hindbrain boundaries express *fgf3* (Mahmood et

al., 1995; Mahmood et al., 1996), and in chick embryos they express *follistatin* (Connolly et al., 1995). Therefore, hindbrain boundaries in all species examined express secreted signalling molecules (or secreted antagonists of signalling molecules), although it appears that the nature of the signal itself may vary between species. Moreover, the effect of the signal may also vary, as there is no evidence of anterior-posterior organisation of neuronal types within segments in chick or mouse.

A new model of boundary formation and maintenance.

The simplest model for the mechanisms which induce boundaries is that boundaries form at segment interfaces, due to interactions that occur only there. For instance, the complementary expression of cell surface ligands and receptors in different segments could underlie signal transduction only at segment interfaces. However, I have found that by blocking the Wnt pathway, most cells in the hindbrain, with the notable exception of r4, can adopt a boundary fate. This implies that boundary cell formation does not necessarily need to be located at segment interfaces. The mechanisms responsible for inducing boundary cells are also active away from interfaces, but are normally prevented from causing ectopic boundary cell formation.

Therefore, a new model can be put forward to describe boundary formation. It is proposed that interactions that occur at interfaces between rhombomeres provide a bias to ensure that boundary cell specification occurs at this location. Secondarily, boundary cells signal to laterally inhibit any further

cells from adopting a boundary fate. Since Wnt1 mediates lateral inhibition only after 18h, this model requires that other factors are involved at earlier stages.

Bibliography.

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *Embo J* **16**, 3797-804.
Adams, K. A., Maida, J. M., Golden, J. A. and Riddle, R. D. (2000). The transcription factor Lmx1b maintains Wnt1 expression within the isthmic organizer. *Development* **127**, 1857-67.
Adamska, M., MacDonald, B. T. and Meisler, M. H. (2003). Doubleridge, a mouse mutant with defective compaction of the apical ectodermal ridge and normal dorsal-ventral patterning of the limb. *Dev Biol* **255**, 350-62.
Ahn, D. G., Ruvinsky, I., Oates, A. C., Silver, L. M. and Ho, R. K. (2000). tbx20, a new vertebrate T-box gene expressed in the cranial motor neurons and developing cardiovascular structures in zebrafish. *Mech Dev* **95**, 253-8.
Akam, M. (1987). The molecular basis for metameric pattern in the Drosophila embryo. *Development* **101**, 1-22.
Allende, M. L. and Weinberg, E. S. (1994). The expression pattern of two zebrafish achaete-scute homolog (ash) genes is altered in the embryonic brain of the cyclops mutant. *Dev Biol* **166**, 509-30.
Andermann, P., Ungos, J. and Raible, D. W. (2002). Neurogenin1 defines zebrafish cranial sensory ganglia precursors. *Dev Biol* **251**, 45-58.
Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6.
Auclair, F., Marchand, R. and Glover, J. C. (1999). Regional patterning of reticulospinal and vestibulospinal neurons in the hindbrain of mouse and rat embryos. *J Comp Neurol* **411**, 288-300.
Barrow, J. R., Stadler, H. S. and Capecchi, M. R. (2000). Roles of Hoxa1 and Hoxa2 in patterning the early hindbrain of the mouse. *Development* **127**, 933-44.
Barrow, J. R., Thomas, K. R., Boussadia-Zahui, O., Moore, R., Kemler, R., Capecchi, M. R. and McMahon, A. P. (2003). Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Dev* **17**, 394-409.
Becker, N., Seitanidou, T., Murphy, P., Mattei, M. G., Topilko, P., Nieto, M. A., Wilkinson, D. G., Charnay, P. and Gilardi-Hebenstreit, P. (1994). Several receptor tyrosine kinase genes of the Eph family are segmentally expressed in the developing hindbrain. *Mech Dev* **47**, 3-17.
Beckers, J., Caron, A., Hrabe de Angelis, M., Hans, S., Campos-Ortega, J. A. and Gossler, A. (2000). Distinct regulatory elements direct delta1 expression in the nervous system and paraxial mesoderm of transgenic mice. *Mech Dev* **95**, 23-34.
Bell, E., Wingate, R. J. and Lumsden, A. (1999). Homeotic transformation of rhombomere identity after localized Hoxb1 misexpression. *Science* **284**, 2168-71.
Bergemann, A. D., Cheng, H. J., Brambilla, R., Klein, R. and Flanagan, J. G. (1995). ELF-2, a new member of the Eph ligand family, is segmentally expressed in mouse embryos in the region of the hindbrain and newly forming somites. *Mol Cell Biol* **15**, 4921-9.

Bermingham, N. A., Hassan, B. A., Wang, V. Y., Fernandez, M., Banfi, S., Bellen, H. J., Fritzsch, B. and Zoghbi, H. Y. (2001). Proprioceptor pathway development is dependent on Math1. *Neuron* **30**, 411-22.
Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat Rev Neurosci* **3**, 517-30.
Bingham, S., Chaudhari, S., Vanderlaan, G., Itoh, M., Chitnis, A. and Chandrasekhar, A. (2003). Neurogenic phenotype of mind bomb mutants leads to severe patterning defects in the zebrafish hindbrain. *Dev Dyn* **228**, 451-63.
Bingham, S., Higashijima, S., Okamoto, H. and Chandrasekhar, A. (2002). The Zebrafish trilobite gene is essential for tangential migration of branchiomotor neurons. *Dev Biol* **242**, 149-60.
Birney, E., Andrews, T. D., Bevan, P., Caccamo, M., Chen, Y., Clarke, L., Coates, G., Cuff, J., Curwen, V., Cutts, T. et al. (2004). An overview of Ensembl. *Genome Res* **14**, 925-8.
Blader, P., Fischer, N., Gradwohl, G., Guillemot, F. and Strahle, U. (1997). The activity of neurogenin1 is controlled by local cues in the zebrafish embryo. *Development* **124**, 4557-69.
Blader, P., Strahle, U. and Ingham, P. W. (1996). Three Wnt genes expressed in a wide variety of tissues during development of the zebrafish, Danio rerio: developmental and evolutionary perspectives. *Dev Genes Evol* **206**, 3-13.
Blair, S. S. (1992). Shaggy (zeste-white 3) and the formation of supernumerary bristle precursors in the developing wing blade of Drosophila. *Dev Biol* **152**, 263-78.
Blair, S. S. (1994). A role for the segment polarity gene shaggy-zeste white 3 in the specification of regional identity in the developing wing of Drosophila. *Dev Biol* **162**, 229-44.
Bray, S. (1998). Notch signalling in Drosophila: three ways to use a pathway. *Semin Cell Dev Biol* **9**, 591-7.
Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol* **11**, 43-9.
Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-45.
Cadigan, K. M. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev* **11**, 3286-305.
Calle-Mustienes Ede, L., Glavic, A., Modolell, J. and Gomez-Skarmeta, J. L. (2002). Xiro homeoproteins coordinate cell cycle exit and primary neuron formation by upregulating neuronal-fate repressors and downregulating the cell-cycle inhibitor XGadd45-gamma. *Mech Dev* **119**, 69.
Candal, E., Thermes, V., Joly, J. S. and Bourrat, F. (2004). Medaka as a model system for the characterisation of cell cycle regulators: a functional analysis of Ol-Gadd45gamma during early embryogenesis. *Mech Dev* **121**, 945-58.
Canfield, V. A., Loppin, B., Thisse, B., Thisse, C., Postlethwait, J. H., Mohideen, M. A., Rajarao, S. J. and Levenson, R. (2002). Na,K-ATPase alpha and beta subunit genes exhibit unique expression patterns during zebrafish embryogenesis. *Mech Dev* **116**, 51-9.
Capdevila, J. and Izpisua Belmonte, J. C. (2001). Patterning mechanisms controlling vertebrate limb development. *Annu Rev Cell Dev Biol* **17**, 87-132.

Carreira-Barbosa, F., Concha, M. L., Takeuchi, M., Ueno, N., Wilson, S. W. and Tada, M. (2003). Prickle 1 regulates cell movements during gastrulation and neuronal migration in zebrafish. *Development* **130**, 4037-46.
Carruthers, S., Mason, J. and Papalopulu, N. (2003). Depletion of the cell-cycle inhibitor p27(Xic1) impairs neuronal differentiation and increases the number of ElrC(+) progenitor cells in Xenopus tropicalis. *Mech Dev* **120**, 607-16.
Casarosa, S., Fode, C. and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. *Development* **126**, 525-34.
Castelo-Branco, G., Wagner, J., Rodriguez, F. J., Kele, J., Sousa, K., Rawal, N., Pasolli, H. A., Fuchs, E., Kitajewski, J. and Arenas, E. (2003). Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. *Proc Natl Acad Sci U S A* **100**, 12747-52.
Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M. and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395**, 604-8.
Cavodeassi, F., Modolell, J. and Gomez-Skarmeta, J. L. (2001). The Iroquois family of genes: from body building to neural patterning. *Development* **128**, 2847-55.
Champagnat, J. and Fortin, G. (1997). Primordial respiratory-like rhythm generation in the vertebrate embryo. *Trends Neurosci* **20**, 119-24.
Chandrasekhar, A. (2004). Turning heads: development of vertebrate branchiomotor neurons. *Dev Dyn* **229**, 143-61.
Chandrasekhar, A., Moens, C. B., Warren, J. T., Jr., Kimmel, C. B. and Kuwada, J. Y. (1997). Development of branchiomotor neurons in zebrafish. *Development* **124**, 2633-44.
Chen, J. and Ruley, H. E. (1998). An enhancer element in the EphA2 (Eck) gene sufficient for rhombomere-specific expression is activated by HOXA1 and HOXB1 homeobox proteins. *J Biol Chem* **273**, 24670-5.
Cheng, Y. C., Amoyel, M., Qiu, X., Jiang, Y. J., Xu, Q. and Wilkinson, D. G. (2004). Notch activation regulates the segregation and differentiation of rhombomere boundary cells in the zebrafish hindbrain. *Dev Cell* **6**, 539-50.
Chenn, A. and Walsh, C. A. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* **297**, 365-9.
Chenn, A. and Walsh, C. A. (2003). Increased neuronal production, enlarged forebrains and cytoarchitectural distortions in beta-catenin overexpressing transgenic mice. *Cereb Cortex* **13**, 599-606.
Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-6.
Chizhikov, V. V. and Millen, K. J. (2005). Roof plate-dependent patterning of the vertebrate dorsal central nervous system. *Dev Biol* **277**, 287-95.
Clarke, J. D. and Lumsden, A. (1993). Segmental repetition of neuronal phenotype sets in the chick embryo hindbrain. *Development* **118**, 151-62.
Connolly, D. J., Patel, K., Seleiro, E. A., Wilkinson, D. G. and Cooke, J. (1995). Cloning, sequencing, and expressional analysis of the chick homologue of follistatin. *Dev Genet* **17**, 65-77.
Cook, M., Gould, A., Brand, N., Davies, J., Strutt, P., Shaknovich, R., Licht, J., Waxman, S., Chen, Z., Gluecksohn-Waelsch, S. et al. (1995). Expression

of the zinc-finger gene PLZF at rhombomere boundaries in the vertebrate hindbrain. *Proc Natl Acad Sci U S A* **92**, 2249-53.
Cooke, J., Moens, C., Roth, L., Durbin, L., Shiomi, K., Brennan, C., Kimmel, C., Wilson, S. and Holder, N. (2001). Eph signalling functions downstream of Val to regulate cell sorting and boundary formation in the caudal hindbrain. *Development* **128**, 571-80.
Cooke, J. E. and Moens, C. B. (2002). Boundary formation in the hindbrain: Eph only it were simple. *Trends Neurosci* **25**, 260-7.
Cordes, S. P. and Barsh, G. S. (1994). The mouse segmentation gene kr encodes a novel basic domain-leucine zipper transcription factor. *Cell* **79**, 1025-34.
Couso, J. P., Bishop, S. A. and Martinez Arias, A. (1994). The wingless signalling pathway and the patterning of the wing margin in Drosophila. *Development* **120**, 621-36.
Cubas, P., de Celis, J. F., Campuzano, S. and Modolell, J. (1991). Proneural clusters of achaete-scute expression and the generation of sensory organs in the Drosophila imaginal wing disc. *Genes Dev* **5**, 996-1008.
Curtin, J. A., Quint, E., Tsipouri, V., Arkell, R. M., Cattanach, B., Copp, A. J., Henderson, D. J., Spurr, N., Stanier, P., Fisher, E. M. et al. (2003). Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse. *Curr Biol* **13**, 1129-33.
Curwen, V., Eyras, E., Andrews, T. D., Clarke, L., Mongin, E., Searle, S. M. and Clamp, M. (2004). The Ensembl automatic gene annotation system. *Genome Res* **14**, 942-50.
Dabdoub, A., Donohue, M. J., Brennan, A., Wolf, V., Montcouquiol, M., Sassoon, D. A., Hseih, J. C., Rubin, J. S., Salinas, P. C. and Kelley, M. W. (2003). Wnt signaling mediates reorientation of outer hair cell stereociliary bundles in the mammalian cochlea. *Development* **130**, 2375-84.
Dahl, D., Rueger, D. C., Bignami, A., Weber, K. and Osborn, M. (1981). Vimentin, the 57 000 molecular weight protein of fibroblast filaments, is the major cytoskeletal component in immature glia. *Eur J Cell Biol* **24**, 191-6.
Dahmann, C. and Basler, K. (1999). Compartment boundaries: at the edge of development. *Trends Genet* **15**, 320-6.
Dale, J. K., Maroto, M., Dequeant, M. L., Malapert, P., McGrew, M. and Pourquie, O. (2003). Periodic notch inhibition by lunatic fringe underlies the chick segmentation clock. *Nature* **421**, 275-8.
Dasen, J. S., Liu, J. P. and Jessell, T. M. (2003). Motor neuron columnar fate imposed by sequential phases of Hox-c activity. *Nature* **425**, 926-33.
Daudet, N. and Lewis, J. (2005). Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. *Development* **132**, 541-51.
Davenne, M., Maconochie, M. K., Neun, R., Pattyn, A., Chambon, P., Krumlauf, R. and Rijli, F. M. (1999). Hoxa2 and Hoxb2 control dorsoventral patterns of neuronal development in the rostral hindbrain. *Neuron* **22**, 677-91.
de Celis, J. F. and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the Drosophila wing. *Development* **124**, 3241-51.
de Celis, J. F. and Bray, S. J. (2000). The Abruptex domain of Notch regulates negative interactions between Notch, its ligands and Fringe. *Development* **127**, 1291-302.

de Celis, J. F. and Garcia-Bellido, A. (1994). Modifications of the notch function by Abruptex mutations in Drosophila melanogaster. *Genetics* **136**, 183-94.
de Celis, J. F., Garcia-Bellido, A. and Bray, S. J. (1996). Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. *Development* **122**, 359-69.
De Robertis, E. M. and Kuroda, H. (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. *Annu Rev Cell Dev Biol* **20**, 285-308.
Desai, A. R. and McConnell, S. K. (2000). Progressive restriction in fate potential by neural progenitors during cerebral cortical development. *Development* **127**, 2863-72.
Di Rocco, G., Mavilio, F. and Zappavigna, V. (1997). Functional dissection of a transcriptionally active, target-specific Hox-Pbx complex. *Embo J* **16**, 3644-54.
Diaz-Benjumea, F. J. and Cohen, S. M. (1995). Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. *Development* **121**, 4215-25.
Dickinson, M. E., Krumlauf, R. and McMahon, A. P. (1994). Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system. *Development* **120**, 1453-71.
Doe, C. Q. and Scott, M. P. (1988). Segmentation and homeotic gene function in the developing nervous system of Drosophila. *Trends Neurosci* **11**, 101-6.
Doherty, D., Feger, G., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1996). Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in Drosophila wing formation. *Genes Dev* **10**, 421-34.
Dolle, P., Lufkin, T., Krumlauf, R., Mark, M., Duboule, D. and Chambon, P. (1993). Local alterations of Krox-20 and Hox gene expression in the hindbrain suggest lack of rhombomeres 4 and 5 in homozygote null Hoxa-1 (Hox-1.6) mutant embryos. *Proc Natl Acad Sci U S A* **90**, 7666-70.
Domingos, P. M., Itasaki, N., Jones, C. M., Mercurio, S., Sargent, M. G., Smith, J. C. and Krumlauf, R. (2001). The Wnt/beta-catenin pathway posteriorizes neural tissue in Xenopus by an indirect mechanism requiring FGF signalling. *Dev Biol* **239**, 148-60.
Dorsky, R. I., Itoh, M., Moon, R. T. and Chitnis, A. (2003). Two tcf3 genes cooperate to pattern the zebrafish brain. *Development* **130**, 1937-1947.
Du, S. J., Purcell, S. M., Christian, J. L., McGrew, L. L. and Moon, R. T. (1995). Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in Xenopus embryos. *Mol Cell Biol* **15**, 2625-34.
Dupe, V., Davenne, M., Brocard, J., Dolle, P., Mark, M., Dierich, A., Chambon, P. and Rijli, F. M. (1997). In vivo functional analysis of the Hoxa-1 3' retinoic acid response element (3'RARE). *Development* **124**, 399-410.
Dupe, V. and Lumsden, A. (2001). Hindbrain patterning involves graded responses to retinoic acid signalling. *Development* **128**, 2199-208.
Echelard, Y., Vassileva, G. and McMahon, A. P. (1994). Cis-acting regulatory sequences governing Wnt-1 expression in the developing mouse CNS. *Development* **120**, 2213-24.
Echevarria, D., Vieira, C., Gimeno, L. and Martinez, S. (2003). Neuroepithelial secondary organizers and cell fate specification in the developing brain. *Brain Res Brain Res Rev* **43**, 179-91.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-80.

Fanto, M. and McNeill, H. (2004). Planar polarity from flies to vertebrates. *J Cell Sci* **117**, 527-33.

Fanto, M., Weber, U., Strutt, D. I. and Mlodzik, M. (2000). Nuclear signaling by Rac and Rho GTPases is required in the establishment of epithelial planar polarity in the Drosophila eye. *Curr Biol* **10**, 979-88.

Feijoo, C. G., Manzanares, M., de la Calle-Mustienes, E., Gomez-Skarmeta, J. L. and Allende, M. L. (2004). The Irx gene family in zebrafish: genomic structure, evolution and initial characterization of irx5b. *Dev Genes Evol* **214**, 277-84.

Ferreiro, B., Skoglund, P., Bailey, A., Dorsky, R. and Harris, W. A. (1993). XASH1, a Xenopus homolog of achaete-scute: a proneural gene in anterior regions of the vertebrate CNS. *Mech Dev* **40**, 25-36.

Fjose, A., Izpisua-Belmonte, J. C., Fromental-Ramain, C. and Duboule, D. (1994). Expression of the zebrafish gene hlx-1 in the prechordal plate and during CNS development. *Development* **120**, 71-81.

Flenniken, A. M., Gale, N. W., Yancopoulos, G. D. and Wilkinson, D. G. (1996). Distinct and overlapping expression patterns of ligands for Eph-related receptor tyrosine kinases during mouse embryogenesis. *Dev Biol* **179**, 382-401.

Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C. and Guillemot, F. (1998). The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* **20**, 483-94.

Frantz, G. D. and McConnell, S. K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. *Neuron* **17**, 55-61.

Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-5.

Frohman, M. A., Martin, G. R., Cordes, S. P., Halamek, L. P. and Barsh, G. S. (1993). Altered rhombomere-specific gene expression and hyoid bone differentiation in the mouse segmentation mutant, kreisler (kr). *Development* **117**, 925-36.

Furthauer, M., Reifers, F., Brand, M., Thisse, B. and Thisse, C. (2001). sprouty4 acts in vivo as a feedback-induced antagonist of FGF signaling in zebrafish. *Development* **128**, 2175-86.

Galceran, J., Sustmann, C., Hsu, S. C., Folberth, S. and Grosschedl, R. (2004). LEF1-mediated regulation of Delta-like1 links Wnt and Notch signaling in somitogenesis. *Genes Dev* **18**, 2718-23.

Gale, N. W., Flenniken, A., Compton, D. C., Jenkins, N., Copeland, N. G., Gilbert, D. J., Davis, S., Wilkinson, D. G. and Yancopoulos, G. D. (1996). Elk-L3, a novel transmembrane ligand for the Eph family of receptor tyrosine kinases, expressed in embryonic floor plate, roof plate and hindbrain segments. *Oncogene* **13**, 1343-52.

Garcia-Bellido, A. (1975). Genetic control of wing disc development in Drosophila. *Ciba Found Symp* **0**, 161-82.

Garcia-Bellido, A., Ripoll, P. and Morata, G. (1973). Developmental compartmentalisation of the wing disk of Drosophila. *Nat New Biol* **245**, 251-3.

Gavalas, A. and Krumlauf, R. (2000). Retinoid signalling and hindbrain patterning. *Curr Opin Genet Dev* **10**, 380-6.

Gavalas, A., Ruhrberg, C., Livet, J., Henderson, C. E. and Krumlauf, R. (2003). Neuronal defects in the hindbrain of Hoxa1, Hoxb1 and Hoxb2 mutants reflect regulatory interactions among these Hox genes. *Development* **130**, 5663-79.

Gavalas, A., Studer, M., Lumsden, A., Rijli, F. M., Krumlauf, R. and Chambon, P. (1998). Hoxa1 and Hoxb1 synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* **125**, 1123-36.

Geling, A., Itoh, M., Tallafuss, A., Chapouton, P., Tannhauser, B., Kuwada, J. Y., Chitnis, A. B. and Bally-Cuif, L. (2003). bHLH transcription factor Her5 links patterning to regional inhibition of neurogenesis at the midbrain-hindbrain boundary. *Development* **130**, 1591-604.

Gendron-Maguire, M., Mallo, M., Zhang, M. and Gridley, T. (1993). Hoxa-2 mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75**, 1317-31.

Giarre, M., Semenov, M. V. and Brown, A. M. (1998). Wnt signaling stabilizes the dual-function protein beta-catenin in diverse cell types. *Ann N Y Acad Sci* **857**, 43-55.

Gilardi-Hebenstreit, P., Nieto, M. A., Frain, M., Mattei, M. G., Chestier, A., Wilkinson, D. G. and Charnay, P. (1992). An Eph-related receptor protein tyrosine kinase gene segmentally expressed in the developing mouse hindbrain. *Oncogene* **7**, 2499-506.

Giles, R. H., van Es, J. H. and Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta* **1653**, 1-24.

Gilland, E. and Baker, R. (1993). Conservation of neuroepithelial and mesodermal segments in the embryonic vertebrate head. *Acta Anat (Basel)* **148**, 110-23.

Giraldez, A. J. and Cohen, S. M. (2003). Wingless and Notch signaling provide cell survival cues and control cell proliferation during wing development. *Development* **130**, 6533-43.

Giudicelli, F., Taillebourg, E., Charnay, P. and Gilardi-Hebenstreit, P. (2001). Krox-20 patterns the hindbrain through both cell-autonomous and non cell-autonomous mechanisms. *Genes Dev* **15**, 567-80.

Glavic, A., Gomez-Skarmeta, J. L. and Mayor, R. (2002). The homeoprotein Xiro1 is required for midbrain-hindbrain boundary formation. *Development* **129**, 1609-21.

Gomez-Skarmeta, J. L., Campuzano, S. and Modolell, J. (2003). Half a century of neural prepatterning: the story of a few bristles and many genes. *Nat Rev Neurosci* **4**, 587-98.

Gomez-Skarmeta, J. L., Diez del Corral, R., de la Calle-Mustienes, E., Ferre-Marco, D. and Modolell, J. (1996). Araucan and caupolican, two members of the novel iroquois complex, encode homeoproteins that control proneural and vein-forming genes. *Cell* **85**, 95-105.

Gomez-Skarmeta, J. L., Glavic, A., de la Calle-Mustienes, E., Modolell, J. and Mayor, R. (1998). Xiro, a Xenopus homolog of the Drosophila Iroquois complex genes, controls development at the neural plate. *Embo J* **17**, 181-90.

Gomez-Skarmeta, J. L. and Modolell, J. (1996). araucan and caupolican provide a link between compartment subdivisions and patterning of sensory organs and veins in the Drosophila wing. *Genes Dev* **10**, 2935-45.

Gotz, M., Hartfuss, E. and Malatesta, P. (2002). Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. *Brain Res Bull* **57**, 777-88.
Gould, A., Itasaki, N. and Krumlauf, R. (1998). Initiation of rhombomeric Hoxb4 expression requires induction by somites and a retinoid pathway. *Neuron* **21**, 39-51.
Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R. and Johnson, J. E. (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31**, 219-32.
Graham, A. and Lumsden, A. (1996). Interactions between rhombomeres modulate Krox-20 and follistatin expression in the chick embryo hindbrain. *Development* **122**, 473-80.
Gräper, L. (1913). Die Rhombomeren und ihre Nervenbeziehungen. *Archiv f. mikr. Anat.* **83**, 371-426.
Greer, J. M., Puetz, J., Thomas, K. R. and Capecchi, M. R. (2000). Maintenance of functional equivalence during paralogous Hox gene evolution. *Nature* **403**, 661-5.
Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M. et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* **66**, 589-600.
Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A. L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**, 463-76.
Guthrie, S., Butcher, M. and Lumsden, A. (1991). Patterns of cell division and interkinetic nuclear migration in the chick embryo hindbrain. *J Neurobiol* **22**, 742-54.
Guthrie, S. and Lumsden, A. (1991). Formation and regeneration of rhombomere boundaries in the developing chick hindbrain. *Development* **112**, 221-9.
Guthrie, S., Prince, V. and Lumsden, A. (1993). Selective dispersal of avian rhombomere cells in orthotopic and heterotopic grafts. *Development* **118**, 527-38.
Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D. and Lewis, J. (1998). Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis. *Development* **125**, 359-70.
Haenlin, M., Kunisch, M., Kramatschek, B. and Campos-Ortega, J. A. (1994). Genomic regions regulating early embryonic expression of the Drosophila neurogenic gene Delta. *Mech Dev* **47**, 99-110.
Hanneman, E., Trevarrow, B., Metcalfe, W. K., Kimmel, C. B. and Westerfield, M. (1988). Segmental pattern of development of the hindbrain and spinal cord of the zebrafish embryo. *Development* **103**, 49-58.
Hans, S. and Campos-Ortega, J. A. (2002). On the organisation of the regulatory region of the zebrafish deltaD gene. *Development* **129**, 4773-84.
Hartenstein, V. (1993). Early pattern of neuronal differentiation in the Xenopus embryonic brainstem and spinal cord. *J Comp Neurol* **328**, 213-31.
Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F. and Kageyama, R. (2004). Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. *Development* **131**, 5539-50.

Heisenberg, C. P. and Tada, M. (2002). Zebrafish gastrulation movements: bridging cell and developmental biology. *Semin Cell Dev Biol* **13**, 471-9.
Heisenberg, C. P., Tada, M., Rauch, G. J., Saude, L., Concha, M. L., Geisler, R., Stemple, D. L., Smith, J. C. and Wilson, S. W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature* **405**, 76-81.
Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P. (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. *Development* **122**, 161-71.
Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of Drosophila. *Cell* **64**, 1083-92.
Hernandez, R. E., Rikhof, H. A., Bachmann, R. and Moens, C. B. (2004). vhnf1 integrates global RA patterning and local FGF signals to direct posterior hindbrain development in zebrafish. *Development* **131**, 4511-20.
Heyman, I., Faissner, A. and Lumsden, A. (1995). Cell and matrix specialisations of rhombomere boundaries. *Dev Dyn* **204**, 301-15.
Heyman, I., Kent, A. and Lumsden, A. (1993). Cellular morphology and extracellular space at rhombomere boundaries in the chick embryo hindbrain. *Dev Dyn* **198**, 241-53.
Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N. and Gotoh, Y. (2004). The Wnt/{beta}-catenin pathway directs neuronal differentiation of cortical neural precursor cells. *Development*.
Hofmann, M., Schuster-Gossler, K., Watabe-Rudolph, M., Aulehla, A., Herrmann, B. G. and Gossler, A. (2004). WNT signaling, in synergy with T/TBX6, controls Notch signaling by regulating Dll1 expression in the presomitic mesoderm of mouse embryos. *Genes Dev* **18**, 2712-7.
Holley, S. A., Geisler, R. and Nusslein-Volhard, C. (2000). Control of her1 expression during zebrafish somitogenesis by a delta-dependent oscillator and an independent wave-front activity. *Genes Dev* **14**, 1678-90.
Holzschuh, J., Barrallo-Gimeno, A., Ettl, A. K., Durr, K., Knapik, E. W. and Driever, W. (2003). Noradrenergic neurons in the zebrafish hindbrain are induced by retinoic acid and require tfap2a for expression of the neurotransmitter phenotype. *Development* **130**, 5741-54.
Huelsken, J. and Behrens, J. (2002). The Wnt signalling pathway. *J Cell Sci* **115**, 3977-8.
Hunt, P., Gulisano, M., Cook, M., Sham, M. H., Faiella, A., Wilkinson, D., Boncinelli, E. and Krumlauf, R. (1991). A distinct Hox code for the branchial region of the vertebrate head. *Nature* **353**, 861-4.
Hynes, M. and Rosenthal, A. (1999). Specification of dopaminergic and serotonergic neurons in the vertebrate CNS. *Curr Opin Neurobiol* **9**, 26-36.
Ikeya, M., Lee, S. M., Johnson, J. E., McMahon, A. P. and Takada, S. (1997). Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* **389**, 966-70.
Ingham, P. W. (1988). The molecular genetics of embryonic pattern formation in Drosophila. *Nature* **335**, 25-34.
Irvine, K. D. and Rauskolb, C. (2001). Boundaries in development: formation and function. *Annu Rev Cell Dev Biol* **17**, 189-214.
Irvine, K. D. and Vogt, T. F. (1997). Dorsal-ventral signaling in limb development. *Curr Opin Cell Biol* **9**, 867-76.

Irvine, K. D. and Wieschaus, E. (1994). fringe, a Boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during Drosophila wing development. *Cell* **79**, 595-606.
Irving, C., Flenniken, A., Alldus, G. and Wilkinson, D. G. (1996a). Cell-cell interactions and segmentation in the developing vertebrate hindbrain. *Biochem Soc Symp* **62**, 85-95.
Irving, C., Nieto, M. A., DasGupta, R., Charnay, P. and Wilkinson, D. G. (1996b). Progressive spatial restriction of Sek-1 and Krox-20 gene expression during hindbrain segmentation. *Dev Biol* **173**, 26-38.
Israsena, N., Hu, M., Fu, W., Kan, L. and Kessler, J. A. (2004). The presence of FGF2 signaling determines whether beta-catenin exerts effects on proliferation or neuronal differentiation of neural stem cells. *Dev Biol* **268**, 220-31.
Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev Cell* **4**, 67-82.
Itoh, M., Kudoh, T., Dedekian, M., Kim, C. H. and Chitnis, A. B. (2002). A role for iro1 and iro7 in the establishment of an anteroposterior compartment of the ectoderm adjacent to the midbrain-hindbrain boundary. *Development* **129**, 2317-27.
Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* **1**, 20-9.
Jessen, J. R., Topczewski, J., Bingham, S., Sepich, D. S., Marlow, F., Chandrasekhar, A. and Solnica-Krezel, L. (2002). Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat Cell Biol* **4**, 610-5.
Jiang, R., Lan, Y., Chapman, H. D., Shawber, C., Norton, C. R., Serreze, D. V., Weinmaster, G. and Gridley, T. (1998). Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* **12**, 1046-57.
Jiang, Y. J., Brand, M., Heisenberg, C. P., Beuchle, D., Furutani-Seiki, M., Kelsh, R. N., Warga, R. M., Granato, M., Haffter, P., Hammerschmidt, M. et al. (1996). Mutations affecting neurogenesis and brain morphology in the zebrafish, Danio rerio. *Development* **123**, 205-16.
Johnston, L. A. and Edgar, B. A. (1998). Wingless and Notch regulate cell-cycle arrest in the developing Drosophila wing. *Nature* **394**, 82-4.
Johnston, S. H., Rauskolb, C., Wilson, R., Prabhakaran, B., Irvine, K. D. and Vogt, T. F. (1997). A family of mammalian Fringe genes implicated in boundary determination and the Notch pathway. *Development* **124**, 2245-54.
Jozefowicz, C., McClintock, J. and Prince, V. (2003). The fates of zebrafish Hox gene duplicates. *J Struct Funct Genomics* **3**, 185-94.
Jungbluth, S., Larsen, C., Wizenmann, A. and Lumsden, A. (2001). Cell mixing between the embryonic midbrain and hindbrain. *Curr Biol* **11**, 204-7.
Kadokawa, Y. and Marunouchi, T. (2002). Chimeric analysis of Notch2 function: a role for Notch2 in the development of the roof plate of the mouse brain. *Dev Dyn* **225**, 126-34.
Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K. and Perrimon, N. (1996). The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes Dev* **10**, 3116-28.

Kan, L., Israsena, N., Zhang, Z., Hu, M., Zhao, L. R., Jalali, A., Sahni, V. and Kessler, J. A. (2004). Sox1 acts through multiple independent pathways to promote neurogenesis. *Dev Biol* **269**, 580-94.
Katbamna, B., Jelaso, A. M. and Ide, C. F. (2004). Connexin 43 expression in glial cells of developing rhombomeres of Xenopus laevis. *Int J Dev Neurosci* **22**, 47-55.
Kawai, H., Arata, N. and Nakayasu, H. (2001). Three-dimensional distribution of astrocytes in zebrafish spinal cord. *Glia* **36**, 406-13.
Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez Esteban, C. and Izpisua Belmonte, J. C. (2001). WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* **104**, 891-900.
Kee, Y. and Bronner-Fraser, M. (2001). Temporally and spatially restricted expression of the helix-loop-helix transcriptional regulator Id1 during avian embryogenesis. *Mech Dev* **109**, 331-5.
Kelly, G. M., Greenstein, P., Erezyilmaz, D. F. and Moon, R. T. (1995). Zebrafish wnt8 and wnt8b share a common activity but are involved in distinct developmental pathways. *Development* **121**, 1787-99.
Kengaku, M., Capdevila, J., Rodriguez-Esteban, C., De La Pena, J., Johnson, R. L., Belmonte, J. C. and Tabin, C. J. (1998). Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science* **280**, 1274-7.
Kiecker, C. and Lumsden, A. (2004). Hedgehog signaling from the ZLI regulates diencephalic regional identity. *Nat Neurosci* **7**, 1242-9.
Kiecker, C. and Niehrs, C. (2001). A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. *Development* **128**, 4189-201.
Kim, C. H., Bae, Y. K., Yamanaka, Y., Yamashita, S., Shimizu, T., Fujii, R., Park, H. C., Yeo, S. Y., Huh, T. L., Hibi, M. et al. (1997). Overexpression of neurogenin induces ectopic expression of HuC in zebrafish. *Neurosci Lett* **239**, 113-6.
Kim, C. H., Oda, T., Itoh, M., Jiang, D., Artinger, K. B., Chandrasekharappa, S. C., Driever, W. and Chitnis, A. B. (2000). Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. *Nature* **407**, 913-6.
Kim, J., Irvine, K. D. and Carroll, S. B. (1995). Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing Drosophila wing. *Cell* **82**, 795-802.
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* **203**, 253-310.
Kimmel, R. A., Turnbull, D. H., Blanquet, V., Wurst, W., Loomis, C. A. and Joyner, A. L. (2000). Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. *Genes Dev* **14**, 1377-89.
Kishi, N., Tang, Z., Maeda, Y., Hirai, A., Mo, R., Ito, M., Suzuki, S., Nakao, K., Kinoshita, T., Kadesch, T. et al. (2001). Murine homologs of deltex define a novel gene family involved in vertebrate Notch signaling and neurogenesis. *Int J Dev Neurosci* **19**, 21-35.
Kontges, G. and Lumsden, A. (1996). Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122**, 3229-42.

Korzh, V., Sleptsova, I., Liao, J., He, J. and Gong, Z. (1998). Expression of zebrafish bHLH genes ngn1 and nrd defines distinct stages of neural differentiation. *Dev Dyn* **213**, 92-104.
Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991). Expression pattern of zebrafish pax genes suggests a role in early brain regionalization. *Nature* **353**, 267-70.
Krumlauf, R. (1992). Evolution of the vertebrate Hox homeobox genes. *Bioessays* **14**, 245-52.
Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.
Kuhl, M. (2004). The WNT/calcium pathway: biochemical mediators, tools and future requirements. *Front Biosci* **9**, 967-74.
Kunisch, M., Haenlin, M. and Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the Drosophila neurogenic gene delta is enhanced by proneural proteins. *Proc Natl Acad Sci U S A* **91**, 10139-43.
Kusumi, K., Dunwoodie, S. L. and Krumlauf, R. (2001). Dynamic expression patterns of the pudgy/spondylocostal dysostosis gene Dll3 in the developing nervous system. *Mech Dev* **100**, 141-4.
Lai, K. O., Chen, Y., Po, H. M., Lok, K. C., Gong, K. and Ip, N. Y. (2004). Identification of the Jak/Stat proteins as novel downstream targets of EphA4 signaling in muscle: implications in the regulation of acetylcholinesterase expression. *J Biol Chem* **279**, 13383-92.
Larsen, C. W., Zeltser, L. M. and Lumsden, A. (2001). Boundary formation and compartition in the avian diencephalon. *J Neurosci* **21**, 4699-711.
Lauderdale, J. D., Davis, N. M. and Kuwada, J. Y. (1997). Axon tracts correlate with netrin-1a expression in the zebrafish embryo. *Mol Cell Neurosci* **9**, 293-313.
Laufer, E., Dahn, R., Orozco, O. E., Yeo, C. Y., Pisenti, J., Henrique, D., Abbott, U. K., Fallon, J. F. and Tabin, C. (1997). Expression of Radical fringe in limb-bud ectoderm regulates apical ectodermal ridge formation. *Nature* **386**, 366-73.
Lawrence, P. A., Casal, J. and Struhl, G. (1999). The hedgehog morphogen and gradients of cell affinity in the abdomen of Drosophila. *Development* **126**, 2441-9.
Lawrence, P. A. and Morata, G. (1994). Homeobox genes: their function in Drosophila segmentation and pattern formation. *Cell* **78**, 181-9.
Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from drosophila? *Cell* **85**, 951-61.
Layer, P. G. and Alber, R. (1990). Patterning of chick brain vesicles as revealed by peanut agglutinin and cholinesterases. *Development* **109**, 613-24.
Lecaudey, V., Anselme, I., Rosa, F. and Schneider-Maunoury, S. (2004). The zebrafish Iroquois gene iro7 positions the r4/r5 boundary and controls neurogenesis in the rostral hindbrain. *Development* **131**, 3121-31.
Lecuit, T. and Cohen, S. M. (1997). Proximal-distal axis formation in the Drosophila leg. *Nature* **388**, 139-45.
Lee, K. J., Dietrich, P. and Jessell, T. M. (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. *Nature* **403**, 734-40.
Lee, K. J. and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu Rev Neurosci* **22**, 261-94.

Lekven, A. C., Buckles, G. R., Kostakis, N. and Moon, R. T. (2003). Wnt1 and wnt10b function redundantly at the zebrafish midbrain-hindbrain boundary. *Dev Biol* **254**, 172-87.
Levitt, P. and Rakic, P. (1980). Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. *J Comp Neurol* **193**, 815-40.
Lewis, E. B. (1978). A gene complex controlling segmentation in Drosophila. *Nature* **276**, 565-70.
Lewis, J. (1998). Notch signalling and the control of cell fate choices in vertebrates. *Semin Cell Dev Biol* **9**, 583-9.
Li, Y. Y., Mi, Z., Feng, Y., McTiernan, C. F., Zhou, R., Robbins, P. D., Watkins, S. C. and Feldman, A. M. (2001). Differential effects of overexpression of two forms of ephrin-A5 on neonatal rat cardiomyocytes. *Am J Physiol Heart Circ Physiol* **281**, H2738-46.
Lin, M. H. and Kopan, R. (2003). Long-range, nonautonomous effects of activated Notch1 on tissue homeostasis in the nail. *Dev Biol* **263**, 343-59.
Livesey, F. J. and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. *Nat Rev Neurosci* **2**, 109-18.
Locy, W. A. (1895). Contribution to the structure and development of the vertebrate head. *Journal of Morphology* **11**, 497-594.
Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20**, 781-810.
Lumsden, A. (1990). The cellular basis of segmentation in the developing hindbrain. *Trends Neurosci* **13**, 329-35.
Lumsden, A. (1999). Closing in on rhombomere boundaries. *Nat Cell Biol* **1**, E83-5.
Lumsden, A. (2004). Segmentation and compartition in the early avian hindbrain. *Mech Dev* **121**, 1081-8.
Lumsden, A., Clarke, J. D., Keynes, R. and Fraser, S. (1994). Early phenotypic choices by neuronal precursors, revealed by clonal analysis of the chick embryo hindbrain. *Development* **120**, 1581-9.
Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-8.
Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-15.
Lumsden, A., Sprawson, N. and Graham, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281-91.
Lyden, D., Young, A. Z., Zagzag, D., Yan, W., Gerald, W., O'Reilly, R., Bader, B. L., Hynes, R. O., Zhuang, Y., Manova, K. et al. (1999). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* **401**, 670-7.
Lyons, D. A., Guy, A. T. and Clarke, J. D. (2003). Monitoring neural progenitor fate through multiple rounds of division in an intact vertebrate brain. *Development* **130**, 3427-36.
Lyu, J., Costantini, F., Jho, E. H. and Joo, C. K. (2003). Ectopic expression of Axin blocks neuronal differentiation of embryonic carcinoma P19 cells. *J Biol Chem* **278**, 13487-95.
Ma, D., Yang, C. H., McNeill, H., Simon, M. A. and Axelrod, J. D. (2003). Fidelity in planar cell polarity signalling. *Nature* **421**, 543-7.

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L. and Anderson, D. J. (1998). neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **20**, 469-82.
MacDonald, B. T., Adamska, M. and Meisler, M. H. (2004). Hypomorphic expression of Dkk1 in the doubleridge mouse: dose dependence and compensatory interactions with Lrp6. *Development* **131**, 2543-52.
Maconochie, M., Nonchev, S., Morrison, A. and Krumlauf, R. (1996). Paralogous Hox genes: function and regulation. *Annu Rev Genet* **30**, 529-56.
Mahmood, R., Kiefer, P., Guthrie, S., Dickson, C. and Mason, I. (1995). Multiple roles for FGF-3 during cranial neural development in the chicken. *Development* **121**, 1399-410.
Mahmood, R., Mason, I. J. and Morriss-Kay, G. M. (1996). Expression of Fgf-3 in relation to hindbrain segmentation, otic pit position and pharyngeal arch morphology in normal and retinoic acid-exposed mouse embryos. *Anat Embryol (Berl)* **194**, 13-22.
Malatesta, P., Hack, M. A., Hartfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F. and Gotz, M. (2003). Neuronal or glial progeny: regional differences in radial glia fate. *Neuron* **37**, 751-64.
Mansouri, A. and Gruss, P. (1998). Pax3 and Pax7 are expressed in commissural neurons and restrict ventral neuronal identity in the spinal cord. *Mech Dev* **78**, 171-8.
Manzanares, M., Bel-Vialar, S., Ariza-McNaughton, L., Ferretti, E., Marshall, H., Maconochie, M. M., Blasi, F. and Krumlauf, R. (2001). Independent regulation of initiation and maintenance phases of Hoxa3 expression in the vertebrate hindbrain involve auto- and cross-regulatory mechanisms. *Development* **128**, 3595-607.
Manzanares, M., Cordes, S., Ariza-McNaughton, L., Sadl, V., Maruthainar, K., Barsh, G. and Krumlauf, R. (1999). Conserved and distinct roles of kreisler in regulation of the paralogous Hoxa3 and Hoxb3 genes. *Development* **126**, 759-69.
Manzanares, M., Cordes, S., Kwan, C. T., Sham, M. H., Barsh, G. S. and Krumlauf, R. (1997). Segmental regulation of Hoxb-3 by kreisler. *Nature* **387**, 191-5.
Marcus, R. C. and Easter, S. S., Jr. (1995). Expression of glial fibrillary acidic protein and its relation to tract formation in embryonic zebrafish (Danio rerio). *J Comp Neurol* **359**, 365-81.
Marin, F. and Charnay, P. (2000). Hindbrain patterning: FGFs regulate Krox20 and mafB/kr expression in the otic/preotic region. *Development* **127**, 4925-35.
Mark, M., Lufkin, T., Vonesch, J. L., Ruberte, E., Olivo, J. C., Dolle, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in Hoxa-1 mutant mice. *Development* **119**, 319-38.
Marshall, H., Morrison, A., Studer, M., Popperl, H. and Krumlauf, R. (1996). Retinoids and Hox genes. *Faseb J* **10**, 969-78.
Marshall, H., Studer, M., Popperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. *Nature* **370**, 567-71.
Martinez, S., Geijo, E., Sanchez-Vives, M. V., Puelles, L. and Gallego, R. (1992). Reduced junctional permeability at interrhombomeric boundaries. *Development* **116**, 1069-76.

Martinez, S., Marin, F., Nieto, M. A. and Puelles, L. (1995). Induction of ectopic engrailed expression and fate change in avian rhombomeres: intersegmental boundaries as barriers. *Mech Dev* **51**, 289-303.
Martinez-Arias, A. and Lawrence, P. A. (1985). Parasegments and compartments in the Drosophila embryo. *Nature* **313**, 639-42.
Mastick, G. S., Fan, C. M., Tessier-Lavigne, M., Serbedzija, G. N., McMahon, A. P. and Easter, S. S., Jr. (1996). Early deletion of neuromeres in Wnt-1-/- mutant mice: evaluation by morphological and molecular markers. *J Comp Neurol* **374**, 246-58.
Matsunaga, E., Katahira, T. and Nakamura, H. (2002). Role of Lmx1b and Wnt1 in mesencephalon and metencephalon development. *Development* **129**, 5269-77.
Maves, L., Jackman, W. and Kimmel, C. B. (2002). FGF3 and FGF8 mediate a rhombomere 4 signaling activity in the zebrafish hindbrain. *Development* **129**, 3825-37.
McClintock, J. M., Carlson, R., Mann, D. M. and Prince, V. E. (2001). Consequences of Hox gene duplication in the vertebrates: an investigation of the zebrafish Hox paralogue group 1 genes. *Development* **128**, 2471-84.
McClintock, J. M., Kheirbek, M. A. and Prince, V. E. (2002). Knockdown of duplicated zebrafish hoxb1 genes reveals distinct roles in hindbrain patterning and a novel mechanism of duplicate gene retention. *Development* **129**, 2339-54.
McClure, B. A. (1891). The segmentation of the primitive vertebrate brain. *Journal of Morphology* **4**, 35-56.
McConnell, S. K. and Kaznowski, C. E. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* **254**, 282-5.
McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.
McKay, I. J., Muchamore, I., Krumlauf, R., Maden, M., Lumsden, A. and Lewis, J. (1994). The kreisler mouse: a hindbrain segmentation mutant that lacks two rhombomeres. *Development* **120**, 2199-211.
McMahon, A. P. and Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-85.
McMahon, A. P., Joyner, A. L., Bradley, A. and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of Wnt-1-/Wnt-1- mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **69**, 581-95.
McMahon, A. P. and Moon, R. T. (1989). Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis. *Cell* **58**, 1075-84.
Meek, A. (1907). The segments of the vertebrate brain and head. *Anatomischer Anzeiger* **31**, 408-415.
Meek, A. (1909). The encephalomeres and cranial nerves of an embryo of Acanthias vulgaris. *Anatomischer Anzeiger* **34**, 473-475.
Meek, A. (1910). The cranial segments and nerves of the rabbit with some remarks on the phylogeny of the nervous system. *Anatomischer Anzeiger* **36**, 560-572.
Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-98.
Meinhardt, H. (1983a). A boundary model for pattern formation in vertebrate limbs. *J Embryol Exp Morphol* **76**, 115-37.

Meinhardt, H. (1983b). Cell determination boundaries as organizing regions for secondary embryonic fields. *Dev Biol* **96**, 375-85.
Mellitzer, G., Xu, Q. and Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.
Mendelson, B. (1986). Development of reticulospinal neurons of the zebrafish. I. Time of origin. *J Comp Neurol* **251**, 160-71.
Metcalfe, W. K., Mendelson, B. and Kimmel, C. B. (1986). Segmental homologies among reticulospinal neurons in the hindbrain of the zebrafish larva. *J Comp Neurol* **251**, 147-59.
Micchelli, C. A., Rulifson, E. J. and Blair, S. S. (1997). The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**, 1485-95.
Mlodzik, M. (2002). Planar cell polarization: do the same mechanisms regulate Drosophila tissue polarity and vertebrate gastrulation? *Trends Genet* **18**, 564-71.
Moens, C. B., Cordes, S. P., Giorgianni, M. W., Barsh, G. S. and Kimmel, C. B. (1998). Equivalence in the genetic control of hindbrain segmentation in fish and mouse. *Development* **125**, 381-91.
Moens, C. B. and Prince, V. E. (2002). Constructing the hindbrain: insights from the zebrafish. *Dev Dyn* **224**, 1-17.
Moens, C. B., Yan, Y. L., Appel, B., Force, A. G. and Kimmel, C. B. (1996). valentino: a zebrafish gene required for normal hindbrain segmentation. *Development* **122**, 3981-90.
Molven, A., Njolstad, P. R. and Fjose, A. (1991). Genomic structure and restricted neural expression of the zebrafish wnt-1 (int-1) gene. *Embo J* **10**, 799-807.
Montcouquiol, M., Rachel, R. A., Lanford, P. J., Copeland, N. G., Jenkins, N. A. and Kelley, M. W. (2003). Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. *Nature* **423**, 173-7.
Moon, R. T., Campbell, R. M., Christian, J. L., McGrew, L. L., Shih, J. and Fraser, S. (1993). Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of Xenopus laevis. *Development* **119**, 97-111.
Moore, K. B., Schneider, M. L. and Vetter, M. L. (2002). Posttranslational mechanisms control the timing of bHLH function and regulate retinal cell fate. *Neuron* **34**, 183-95.
Moriguchi, T., Kawachi, K., Kamakura, S., Masuyama, N., Yamanaka, H., Matsumoto, K., Kikuchi, A. and Nishida, E. (1999). Distinct domains of mouse dishevelled are responsible for the c-Jun N-terminal kinase/stress-activated protein kinase activation and the axis formation in vertebrates. *J Biol Chem* **274**, 30957-62.
Moriyoshi, K., Richards, L. J., Akazawa, C., O'Leary, D. D. and Nakanishi, S. (1996). Labeling neural cells using adenoviral gene transfer of membrane-targeted GFP. *Neuron* **16**, 255-60.
Muller, T., Brohmann, H., Pierani, A., Heppenstall, P. A., Lewin, G. R., Jessell, T. M. and Birchmeier, C. (2002). The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. *Neuron* **34**, 551-62.
Munchberg, S. R., Ober, E. A. and Steinbeisser, H. (1999). Expression of the Ets transcription factors erm and pea3 in early zebrafish development. *Mech Dev* **88**, 233-6.

Muroyama, Y., Fujihara, M., Ikeya, M., Kondoh, H. and Takada, S. (2002). Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. *Genes Dev* **16**, 548-53.
Muroyama, Y., Kondoh, H. and Takada, S. (2004). Wnt proteins promote neuronal differentiation in neural stem cell culture. *Biochem Biophys Res Commun* **313**, 915-21.
Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. *Dev Biol* **174**, 233-47.
Nakamura, Y., Nishisho, I., Kinzler, K. W., Vogelstein, B., Miyoshi, Y., Miki, Y., Ando, H., Horii, A. and Nagase, H. (1991). Mutations of the adenomatous polyposis coli gene in familial polyposis coli patients and sporadic colorectal tumors. *Princess Takamatsu Symp* **22**, 285-92.
Neal, H. V. (1918). Neuromeres and metameres. *Journal of Morphology* **31**, 293-315.
Neave, B., Rodaway, A., Wilson, S. W., Patient, R. and Holder, N. (1995). Expression of zebrafish GATA 3 (gta3) during gastrulation and neurulation suggests a role in the specification of cell fate. *Mech Dev* **51**, 169-82.
Nelson, W. J. and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483-7.
Neumann, C. J. and Cohen, S. M. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. *Development* **124**, 871-80.
Nguyen, V., Candal Suarez, E. M., Sharif, A., Joly, J. S. and Bourrat, F. (2001). Expression of Ol-KIP, a cyclin-dependent kinase inhibitor, in embryonic and adult medaka (Oryzias latipes) central nervous system. *Dev Dyn* **222**, 439-49.
Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dolle, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127**, 75-85.
Nielsen, A. L. and Jorgensen, A. L. (2003). Structural and functional characterization of the zebrafish gene for glial fibrillary acidic protein, GFAP. *Gene* **310**, 123-32.
Nieto, M., Schuurmans, C., Britz, O. and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. *Neuron* **29**, 401-13.
Nieto, M. A., Gilardi-Hebenstreit, P., Charnay, P. and Wilkinson, D. G. (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* **116**, 1137-50.
Nittenberg, R., Patel, K., Joshi, Y., Krumlauf, R., Wilkinson, D. G., Brickell, P. M., Tickle, C. and Clarke, J. D. (1997). Cell movements, neuronal organisation and gene expression in hindbrains lacking morphological boundaries. *Development* **124**, 2297-306.
Noctor, S. C., Flint, A. C., Weissman, T. A., Wong, W. S., Clinton, B. K. and Kriegstein, A. R. (2002). Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J Neurosci* **22**, 3161-73.
Noden, D. M. (1983). The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Dev Biol* **96**, 144-65.

Noden, D. M. (1988). Interactions and fates of avian craniofacial mesenchyme. *Development* **103 Suppl**, 121-40.
Nonchev, S., Vesque, C., Maconochie, M., Seitanidou, T., Ariza-McNaughton, L., Frain, M., Marshall, H., Sham, M. H., Krumlauf, R. and Charnay, P. (1996). Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20. *Development* **122**, 543-54.
Noordermeer, J., Klingensmith, J., Perrimon, N. and Nusse, R. (1994). dishevelled and armadillo act in the wingless signalling pathway in Drosophila. *Nature* **367**, 80-3.
Nordlander, R. H., Baden, S. T. and Ryba, T. M. (1985). Development of early brainstem projections to the tail spinal cord of Xenopus. *J Comp Neurol* **231**, 519-29.
Nordstrom, U., Jessell, T. M. and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. *Nat Neurosci* **5**, 525-32.
Nusse, R., Brown, A., Papkoff, J., Scambler, P., Shackleford, G., McMahon, A., Moon, R. and Varmus, H. (1991). A new nomenclature for int-1 and related genes: the Wnt gene family. *Cell* **64**, 231.
Nusse, R. and Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**, 99-109.
Nusslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature* **287**, 795-801.
Odenthal, J. and Nusslein-Volhard, C. (1998). fork head domain genes in zebrafish. *Dev Genes Evol* **208**, 245-58.
Ohnuma, S., Philpott, A., Wang, K., Holt, C. E. and Harris, W. A. (1999). p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. *Cell* **99**, 499-510.
Orr, H. (1887). Contribution to the embryology of the lizard; with especial reference to the central nervous system and some organs of the head; together with observations on the origins of vertebrates. *Journal of Morphology* **1**, 311-372.
Otero, J. J., Fu, W., Kan, L., Cuadra, A. E. and Kessler, J. A. (2004). Beta-catenin signaling is required for neural differentiation of embryonic stem cells. *Development* **131**, 3545-57.
Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Res* **21**, 1087-95.
Packer, A. I., Crotty, D. A., Elwell, V. A. and Wolgemuth, D. J. (1998). Expression of the murine Hoxa4 gene requires both autoregulation and a conserved retinoic acid response element. *Development* **125**, 1991-8.
Pandur, P., Lasche, M., Eisenberg, L. M. and Kuhl, M. (2002). Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* **418**, 636-41.
Panhuysen, M., Vogt Weisenhorn, D. M., Blanquet, V., Brodski, C., Heinzmann, U., Beisker, W. and Wurst, W. (2004). Effects of Wnt1 signaling on proliferation in the developing mid-/hindbrain region. *Mol Cell Neurosci* **26**, 101-11.
Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-12.

Papalopulu, N., Lovell-Badge, R. and Krumlauf, R. (1991). The expression of murine Hox-2 genes is dependent on the differentiation pathway and displays a collinear sensitivity to retinoic acid in F9 cells and Xenopus embryos. *Nucleic Acids Res* **19**, 5497-506.
Park, H. C. and Appel, B. (2003). Delta-Notch signaling regulates oligodendrocyte specification. *Development* **130**, 3747-55.
Park, H. C., Hong, S. K., Kim, H. S., Kim, S. H., Yoon, E. J., Kim, C. H., Miki, N. and Huh, T. L. (2000a). Structural comparison of zebrafish Elav/Hu and their differential expressions during neurogenesis. *Neurosci Lett* **279**, 81-4.
Park, H. C., Kim, C. H., Bae, Y. K., Yeo, S. Y., Kim, S. H., Hong, S. K., Shin, J., Yoo, K. W., Hibi, M., Hirano, T. et al. (2000b). Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. *Dev Biol* **227**, 279-93.
Park, H. C., Mehta, A., Richardson, J. S. and Appel, B. (2002). olig2 is required for zebrafish primary motor neuron and oligodendrocyte development. *Dev Biol* **248**, 356-68.
Pasini, A. and Wilkinson, D. G. (2002). Stabilizing the regionalisation of the developing vertebrate central nervous system. *Bioessays* **24**, 427-38.
Pattyn, A., Vallstedt, A., Dias, J. M., Samad, O. A., Krumlauf, R., Rijli, F. M., Brunet, J. F. and Ericson, J. (2003a). Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors. *Genes Dev* **17**, 729-37.
Pattyn, A., Vallstedt, A., Dias, J. M., Sander, M. and Ericson, J. (2003b). Complementary roles for Nkx6 and Nkx2 class proteins in the establishment of motoneuron identity in the hindbrain. *Development* **130**, 4149-59.
Payre, F., Vincent, A. and Carreno, S. (1999). ovo/svb integrates Wingless and DER pathways to control epidermis differentiation. *Nature* **400**, 271-5.
Pearson, B. J. and Doe, C. Q. (2003). Regulation of neuroblast competence in Drosophila. *Nature* **425**, 624-8.
Pearson, B. J. and Doe, C. Q. (2004). Specification of temporal identity in the developing nervous system. *Annu Rev Cell Dev Biol* **20**, 619-47.
Peel, A. (2004). The evolution of arthropod segmentation mechanisms. *Bioessays* **26**, 1108-16.
Phillips, R. G. and Whittle, J. R. (1993). wingless expression mediates determination of peripheral nervous system elements in late stages of Drosophila wing disc development. *Development* **118**, 427-38.
Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-15.
Pinto, D., Gregorieff, A., Begthel, H. and Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev* **17**, 1709-13.
Polakis, P. (1999). The oncogenic activation of beta-catenin. *Curr Opin Genet Dev* **9**, 15-21.
Poliakov, A., Cotrina, M. and Wilkinson, D. G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Dev Cell* **7**, 465-80.
Popperl, H., Bienz, M., Studer, M., Chan, S. K., Aparicio, S., Brenner, S., Mann, R. S. and Krumlauf, R. (1995). Segmental expression of Hoxb-1 is

controlled by a highly conserved autoregulatory loop dependent upon exd/pbx. *Cell* **81**, 1031-42.
Popperl, H. and Featherstone, M. S. (1992). An autoregulatory element of the murine Hox-4.2 gene. *Embo J* **11**, 3673-80.
Pourquie, O. (2001). Vertebrate somitogenesis. *Annu Rev Cell Dev Biol* **17**, 311-50.
Price, S. R., De Marco Garcia, N. V., Ranscht, B. and Jessell, T. M. (2002). Regulation of motor neuron pool sorting by differential expression of type II cadherins. *Cell* **109**, 205-16.
Prince, V. E., Holley, S. A., Bally-Cuif, L., Prabhakaran, B., Oates, A. C., Ho, R. K. and Vogt, T. F. (2001). Zebrafish lunatic fringe demarcates segmental boundaries. *Mech Dev* **105**, 175-80.
Prince, V. E., Moens, C. B., Kimmel, C. B. and Ho, R. K. (1998). Zebrafish hox genes: expression in the hindbrain region of wild-type and mutants of the segmentation gene, valentino. *Development* **125**, 393-406.
Pritz, M. B. (1999). Rhombomere development in a reptilian embryo. *J Comp Neurol* **411**, 317-26.
Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S. L., Wurst, W. and Simeone, A. (2004). Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* **131**, 2037-48.
Qiu, X., Xu, H., Haddon, C., Lewis, J. and Jiang, Y. J. (2004). Sequence and embryonic expression of three zebrafish fringe genes: lunatic fringe, radical fringe, and manic fringe. *Dev Dyn* **231**, 621-30.
Raible, F. and Brand, M. (2001). Tight transcriptional control of the ETS domain factors Erm and Pea3 by Fgf signaling during early zebrafish development. *Mech Dev* **107**, 105-17.
Rakic, P. (1978). Neuronal migration and contact guidance in the primate telencephalon. *Postgrad Med J* **54 Suppl 1**, 25-40.
Ramain, P., Khechumian, K., Seugnet, L., Arbogast, N., Ackermann, C. and Heitzler, P. (2001). Novel Notch alleles reveal a Deltex-dependent pathway repressing neural fate. *Curr Biol* **11**, 1729-38.
Rauch, G. J., Hammerschmidt, M., Blader, P., Schauerte, H. E., Strahle, U., Ingham, P. W., McMahon, A. P. and Haffter, P. (1997). Wnt5 is required for tail formation in the zebrafish embryo. *Cold Spring Harb Symp Quant Biol* **62**, 227-34.
Richardson, W. D., Smith, H. K., Sun, T., Pringle, N. P., Hall, A. and Woodruff, R. (2000). Oligodendrocyte lineage and the motor neuron connection. *Glia* **29**, 136-42.
Rijli, F. M., Mark, M., Lakkaraju, S., Dierich, A., Dolle, P. and Chambon, P. (1993). A homeotic transformation is generated in the rostral branchial region of the head by disruption of Hoxa-2, which acts as a selector gene. *Cell* **75**, 1333-49.
Riley, B. B., Chiang, M. Y., Storch, E. M., Heck, R., Buckles, G. R. and Lekven, A. C. (2004). Rhombomere boundaries are Wnt signaling centers that regulate metameric patterning in the zebrafish hindbrain. *Dev Dyn* **231**, 278-91.
Rodriguez-Esteban, C., Schwabe, J. W., De La Pena, J., Foys, B., Eshelman, B. and Belmonte, J. C. (1997). Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. *Nature* **386**, 360-6.

Roehl, H. and Nusslein-Volhard, C. (2001). Zebrafish pea3 and erm are general targets of FGF8 signaling. *Curr Biol* **11**, 503-7.
Roose, J. and Clevers, H. (1999). TCF transcription factors: molecular switches in carcinogenesis. *Biochim Biophys Acta* **1424**, M23-37.
Ross, S. E., Greenberg, M. E. and Stiles, C. D. (2003). Basic helix-loop-helix factors in cortical development. *Neuron* **39**, 13-25.
Rowitch, D. H., Lu, Q. R., Kessaris, N. and Richardson, W. D. (2002). An 'oligarchy' rules neural development. *Trends Neurosci* **25**, 417-22.
Rulifson, E. J. and Blair, S. S. (1995). Notch regulates wingless expression and is not required for reception of the paracrine wingless signal during wing margin neurogenesis in Drosophila. *Development* **121**, 2813-24.
Rulifson, E. J., Micchelli, C. A., Axelrod, J. D., Perrimon, N. and Blair, S. S. (1996). wingless refines its own expression domain on the Drosophila wing margin. *Nature* **384**, 72-4.
Samad, O. A., Geisen, M. J., Caronia, G., Varlet, I., Zappavigna, V., Ericson, J., Goridis, C. and Rijli, F. M. (2004). Integration of anteroposterior and dorsoventral regulation of Phox2b transcription in cranial motoneuron progenitors by homeodomain proteins. *Development* **131**, 4071-83.
Sanchez-Camacho, C., Martin, O., Ten Donkelaar, H. J. and Gonzalez, A. (2002). Descending supraspinal pathways in amphibians: III. Development of descending projections to the spinal cord in Xenopus laevis with emphasis on the catecholaminergic inputs. *J Comp Neurol* **446**, 11-24.
Sancho, E., Batlle, E. and Clevers, H. (2003). Live and let die in the intestinal epithelium. *Curr Opin Cell Biol* **15**, 763-70.
Sanson, B. (2001). Generating patterns from fields of cells. Examples from Drosophila segmentation. *EMBO Rep* **2**, 1083-8.
Sanson, B., Alexandre, C., Fascetti, N. and Vincent, J. P. (1999). Engrailed and hedgehog make the range of Wingless asymmetric in Drosophila embryos. *Cell* **98**, 207-16.
Schilling, T. F., Prince, V. and Ingham, P. W. (2001). Plasticity in zebrafish hox expression in the hindbrain and cranial neural crest. *Dev Biol* **231**, 201-16.
Schneider-Maunoury, S., Seitanidou, T., Charnay, P. and Lumsden, A. (1997). Segmental and neuronal architecture of the hindbrain of Krox-20 mouse mutants. *Development* **124**, 1215-26.
Schneider-Maunoury, S., Topilko, P., Seitandou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C. and Charnay, P. (1993). Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75**, 1199-214.
Schwarz, Q., Gu, C., Fujisawa, H., Sabelko, K., Gertsenstein, M., Nagy, A., Taniguchi, M., Kolodkin, A. L., Ginty, D. D., Shima, D. T. et al. (2004). Vascular endothelial growth factor controls neuronal migration and cooperates with Sema3A to pattern distinct compartments of the facial nerve. *Genes Dev* **18**, 2822-34.
Schweisguth, F. (2004). Notch signaling activity. *Curr Biol* **14**, R129-38.
Scott, M. P. and Carroll, S. B. (1987). The segmentation and homeotic gene network in early Drosophila development. *Cell* **51**, 689-98.
Seo, H. C., Saetre, B. O., Havik, B., Ellingsen, S. and Fjose, A. (1998). The zebrafish Pax3 and Pax7 homologues are highly conserved, encode multiple isoforms and show dynamic segment-like expression in the developing brain. *Mech Dev* **70**, 49-63.

Sham, M. H., Vesque, C., Nonchev, S., Marshall, H., Frain, M., Gupta, R. D., Whiting, J., Wilkinson, D., Charnay, P. and Krumlauf, R. (1993). The zinc finger gene Krox20 regulates HoxB2 (Hox2.8) during hindbrain segmentation. *Cell* **72**, 183-96.

Sharma, M. K., Denovan-Wright, E. M., Boudreau, M. E. and Wright, J. M. (2003). A cellular retinoic acid-binding protein from zebrafish (Danio rerio): cDNA sequence, phylogenetic analysis, mRNA expression, and gene linkage mapping. *Gene* **311**, 119-28.

Sharma, R. P. and Chopra, V. L. (1976). Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. *Dev Biol* **48**, 461-5.

Sharpe, C., Lawrence, N. and Martinez Arias, A. (2001). Wnt signalling: a theme with nuclear variations. *Bioessays* **23**, 311-8.

Sheldahl, L. C., Slusarski, D. C., Pandur, P., Miller, J. R., Kuhl, M. and Moon, R. T. (2003). Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. *J Cell Biol* **161**, 769-77.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A* **96**, 5522-7.

Simpson, P. (1997). Notch signalling in development: on equivalence groups and asymmetric developmental potential. *Curr Opin Genet Dev* **7**, 537-42.

Simpson, P., El Messal, M., Moscoso del Prado, J. and Ripoll, P. (1988). Stripes of positional homologies across the wing blade of Drosophila melanogaster. *Development* **103**, 391-401.

Skeath, J. B. and Carroll, S. B. (1991). Regulation of achaete-scute gene expression and sensory organ pattern formation in the Drosophila wing. *Genes Dev* **5**, 984-95.

Skeath, J. B. and Carroll, S. B. (1994). The achaete-scute complex: generation of cellular pattern and fate within the Drosophila nervous system. *Faseb J* **8**, 714-21.

Soshnikova, N., Zechner, D., Huelsken, J., Mishina, Y., Behringer, R. R., Taketo, M. M., Crenshaw, E. B., 3rd and Birchmeier, W. (2003). Genetic interaction between Wnt/beta-catenin and BMP receptor signaling during formation of the AER and the dorsal-ventral axis in the limb. *Genes Dev* **17**, 1963-8.

Sprague, J., Clements, D., Conlin, T., Edwards, P., Frazer, K., Schaper, K., Segerdell, E., Song, P., Sprunger, B. and Westerfield, M. (2003). The Zebrafish Information Network (ZFIN): the zebrafish model organism database. *Nucleic Acids Res* **31**, 241-3.

St Johnston, D. and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. *Cell* **68**, 201-19.

Stevens, C. B., Davies, A. L., Battista, S., Lewis, J. H. and Fekete, D. M. (2003). Forced activation of Wnt signaling alters morphogenesis and sensory organ identity in the chicken inner ear. *Dev Biol* **261**, 149-64.

Streeter, G. L. (1908). The nuclei of origin of the cranial nerves in the 10 mm. human embryo. *Anatomical Record* **2**, 111-115.

Strigini, M. and Cohen, S. M. (1999). Formation of morphogen gradients in the Drosophila wing. *Semin Cell Dev Biol* **10**, 335-44.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L., Rijli, F. M., Chambon, P. and Krumlauf, R. (1998). Genetic interactions between Hoxa1

and Hoxb1 reveal new roles in regulation of early hindbrain patterning. *Development* **125**, 1025-36.
Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A. and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb-1. *Nature* **384**, 630-4.
Swiatek, P. J. and Gridley, T. (1993). Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. *Genes Dev* **7**, 2071-84.
Tada, M., Concha, M. L. and Heisenberg, C. P. (2002). Non-canonical Wnt signalling and regulation of gastrulation movements. *Semin Cell Dev Biol* **13**, 251-60.
Takahashi, M. and Osumi, N. (2002). Pax6 regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains. *Development* **129**, 1327-38.
Takasu, M. A., Dalva, M. B., Zigmond, R. E. and Greenberg, M. E. (2002). Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. *Science* **295**, 491-5.
Tan, J. T., Korzh, V. and Gong, Z. (1999). Expression of a zebrafish iroquois homeobox gene, Ziro3, in the midline axial structures and central nervous system. *Mech Dev* **87**, 165-8.
Taneja, R., Thisse, B., Rijli, F. M., Thisse, C., Bouillet, P., Dolle, P. and Chambon, P. (1996). The expression pattern of the mouse receptor tyrosine kinase gene MDK1 is conserved through evolution and requires Hoxa-2 for rhombomere-specific expression in mouse embryos. *Dev Biol* **177**, 397-412.
Tang, K., Yang, J., Gao, X., Wang, C., Liu, L., Kitani, H., Atsumi, T. and Jing, N. (2002). Wnt-1 promotes neuronal differentiation and inhibits gliogenesis in P19 cells. *Biochem Biophys Res Commun* **293**, 167-73.
Tautz, D. (2004). Segmentation. *Dev Cell* **7**, 301-12.
Tavares, A. T., Tsukui, T. and Izpisua Belmonte, J. C. (2000). Evidence that members of the Cut/Cux/CDP family may be involved in AER positioning and polarizing activity during chick limb development. *Development* **127**, 5133-44.
Tetsu, O. and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422-6.
Thaeron, C., Avaron, F., Casane, D., Borday, V., Thisse, B., Thisse, C., Boulekbache, H. and Laurenti, P. (2000). Zebrafish evx1 is dynamically expressed during embryogenesis in subsets of interneurones, posterior gut and urogenital system. *Mech Dev* **99**, 167-72.
Theil, T., Frain, M., Gilardi-Hebenstreit, P., Flenniken, A., Charnay, P. and Wilkinson, D. G. (1998). Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20. *Development* **125**, 443-52.
Theodorakis, K., Kyriakopoulou, K., Wassef, M. and Karagogeos, D. (2002). Novel sites of expression of the bHLH gene NSCL1 in the developing nervous system. *Mech Dev* **119 Suppl 1**, S103-6.
Tolwinski, N. S. and Wieschaus, E. (2004). Rethinking WNT signaling. *Trends Genet* **20**, 177-81.
Toresson, H., Parmar, M. and Campbell, K. (2000). Expression of Meis and Pbx genes and their protein products in the developing telencephalon: implications for regional differentiation. *Mech Dev* **94**, 183-7.

Trainor, P. and Krumlauf, R. (2000a). Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm. *Nat Cell Biol* **2**, 96-102.
Trainor, P. A. and Krumlauf, R. (2000b). Patterning the cranial neural crest: hindbrain segmentation and Hox gene plasticity. *Nat Rev Neurosci* **1**, 116-24.
Trainor, P. A. and Krumlauf, R. (2001). Hox genes, neural crest cells and branchial arch patterning. *Curr Opin Cell Biol* **13**, 698-705.
Trevarrow, B., Marks, D. L. and Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron* **4**, 669-79.
Vaage, S. (1969). The segmentation of the primitive neural tube in chick embryos (Gallus domesticus). A morphological, histochemical and autoradiographical investigation. *Ergeb Anat Entwicklungsgesch* **41**, 3-87.
Vallstedt, A., Klos, J. M. and Ericson, J. (2005). Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. *Neuron* **45**, 55-67.
van Mier, P. and ten Donkelaar, H. J. (1984). Early development of descending pathways from the brain stem to the spinal cord in Xenopus laevis. *Anat Embryol (Berl)* **170**, 295-306.
van Mier, P. and ten Donkelaar, H. J. (1989). Structural and functional properties of reticulospinal neurons in the early-swimming stage Xenopus embryo. *J Neurosci* **9**, 25-37.
van Ooyen, A. and Nusse, R. (1984). Structure and nucleotide sequence of the putative mammary oncogene int-1; proviral insertions leave the protein-encoding domain intact. *Cell* **39**, 233-40.
Veeman, M. T., Axelrod, J. D. and Moon, R. T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell* **5**, 367-77.
Vesque, C., Maconochie, M., Nonchev, S., Ariza-McNaughton, L., Kuroiwa, A., Charnay, P. and Krumlauf, R. (1996). Hoxb-2 transcriptional activation in rhombomeres 3 and 5 requires an evolutionarily conserved cis-acting element in addition to the Krox-20 binding site. *Embo J* **15**, 5383-96.
Wakamatsu, Y. and Weston, J. A. (1997). Sequential expression and role of Hu RNA-binding proteins during neurogenesis. *Development* **124**, 3449-60.
Walshe, J., Maroon, H., McGonnell, I. M., Dickson, C. and Mason, I. (2002). Establishment of Hindbrain Segmental Identity Requires Signaling by FGF3 and FGF8. *Curr Biol* **12**, 1117-23.
Wan, S., Cato, A. M. and Skaer, H. (2000). Multiple signalling pathways establish cell fate and cell number in Drosophila malpighian tubules. *Dev Biol* **217**, 153-65.
Wang, X., Emelyanov, A., Sleptsova-Friedrich, I., Korzh, V. and Gong, Z. (2001). Expression of two novel zebrafish iroquois homologues (ziro1 and ziro5) during early development of axial structures and central nervous system. *Mech Dev* **105**, 191-5.
Waskiewicz, A. J., Rikhof, H. A. and Moens, C. B. (2002). Eliminating zebrafish pbx proteins reveals a hindbrain ground state. *Dev Cell* **3**, 723-33.
Weber, U., Paricio, N. and Mlodzik, M. (2000). Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. *Development* **127**, 3619-29.
Westerfield, M. (1993). The Zebrafish Book. Eugene: University of Oregon Press.

Wiellette, E. L. and Sive, H. (2003). vhnf1 and Fgf signals synergize to specify rhombomere identity in the zebrafish hindbrain. *Development* **130**, 3821-9.
Wilkinson, D. G. (1995). Genetic control of segmentation in the vertebrate hindbrain. *Perspect Dev Neurobiol* **3**, 29-38.
Wilkinson, D. G., Bailes, J. A. and McMahon, A. P. (1987). Expression of the proto-oncogene int-1 is restricted to specific neural cells in the developing mouse embryo. *Cell* **50**, 79-88.
Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charnay, P. (1989a). Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. *Nature* **337**, 461-4.
Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R. (1989b). Segmental expression of Hox-2 homoeobox-containing genes in the developing mouse hindbrain. *Nature* **341**, 405-9.
Wingate, R. J. and Lumsden, A. (1996). Persistence of rhombomeric organisation in the postsegmental hindbrain. *Development* **122**, 2143-52.
Wizenmann, A. and Lumsden, A. (1997). Segregation of rhombomeres by differential chemoaffinity. *Mol Cell Neurosci* **9**, 448-59.
Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J Theor Biol* **25**, 1-47.
Wurst, W. and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of the isthmic organizer. *Nat Rev Neurosci* **2**, 99-108.
Xu, Q., Alldus, G., Holder, N. and Wilkinson, D. G. (1995). Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the Xenopus and zebrafish hindbrain. *Development* **121**, 4005-16.
Xu, Q., Holder, N., Patient, R. and Wilson, S. W. (1994). Spatially regulated expression of three receptor tyrosine kinase genes during gastrulation in the zebrafish. *Development* **120**, 287-99.
Xu, Q., Mellitzer, G., Robinson, V. and Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-71.
Xu, Q., Mellitzer, G. and Wilkinson, D. G. (2000). Roles of Eph receptors and ephrins in segmental patterning. *Philos Trans R Soc Lond B Biol Sci* **355**, 993-1002.
Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S. and Nishida, E. (2002). JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep* **3**, 69-75.
Yan, S. J., Gu, Y., Li, W. X. and Fleming, R. J. (2004). Multiple signaling pathways and a selector protein sequentially regulate Drosophila wing development. *Development* **131**, 285-98.
Yarden, A., Salomon, D. and Geiger, B. (1995). Zebrafish cyclin D1 is differentially expressed during early embryogenesis. *Biochim Biophys Acta* **1264**, 257-60.
Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-66.
Yoshida, M. (2001). Glial-defined boundaries in Xenopus CNS. *Dev Neurosci* **23**, 299-306.

Yoshida, M. and Colman, D. R. (2000). Glial-defined rhombomere boundaries in developing Xenopus hindbrain. *J Comp Neurol* **424**, 47-57.
Zechner, D., Fujita, Y., Hulsken, J., Muller, T., Walther, I., Taketo, M. M., Crenshaw, E. B., 3rd, Birchmeier, W. and Birchmeier, C. (2003). beta-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. *Dev Biol* **258**, 406-18.
Zeltser, L. M., Larsen, C. W. and Lumsden, A. (2001). A new developmental compartment in the forebrain regulated by Lunatic fringe. *Nat Neurosci* **4**, 683-4.
Zhai, L., Chaturvedi, D. and Cumberledge, S. (2004). Drosophila wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine. *J Biol Chem* **279**, 33220-7.
Zupanc, G. K. and Clint, S. C. (2003). Potential role of radial glia in adult neurogenesis of teleost fish. *Glia* **43**, 77-86.

Appendix A : "Wnt1 regulates neurogenesis and mediates lateral inhibition of boundary cell specification in the zebrafish hindbrain", Amoyel* et al., *2005.

Wnt1 regulates neurogenesis and mediates lateral inhibition of boundary cell specification in the zebrafish hindbrain

Marc Amoyel[1], Yi-Chuan Cheng[1], Yun-Jin Jiang[2] and David G. Wilkinson[1,*]

Development

Appendix B : "Notch activation regulates the segregation and differentiation of rhombomere boundary cells in the zebrafish hindbrain", Cheng* et al., *2004.

Developmental Cell, Vol. 6, 539–550, April, 2004, Copyright ©2004 by Cell Press

Notch Activation Regulates the Segregation and Differentiation of Rhombomere Boundary Cells in the Zebrafish Hindbrain

Yi-Chuan Cheng,[1] Marc Amoyel,[1]
Xuehui Qiu,[3] Yun-Jin Jiang,[3]
Qiling Xu,[2] and David G. Wilkinson[1,*]