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

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Summary

The formation of localised signalling centres is essential for patterning of a number of tissues during development. Previous work has revealed that a distinct population of boundary cells forms at the interface of segments in the vertebrate hindbrain, but the role of these cells is not known. We have investigated the function of the Wnt1 signalling molecule that is expressed by boundary and roof plate cells in the zebrafish hindbrain. Knockdown of *wnt1* or of *tcf3b*, a mediator of Wnt signalling, leads to ectopic expression of boundary cell markers, *rfng* and *foxb1.2,* in non-boundary regions of the hindbrain. Ectopic boundary marker expression also occurs following knockdown of *rfng*, a modulator of Notch signalling required for *wnt1* expression at hindbrain boundaries. We show that the boundary and roof plate expression of *wnt1* each contribute to upregulation of proneural and *delta* gene expression and neurogenesis in non-boundary regions, which in turn blocks ectopic boundary marker expression. Boundary cells therefore play a key role in the regulation of cell differentiation in the zebrafish hindbrain. The network of genes underlying the regulation of neurogenesis and lateral inhibition of boundary cell formation by Wnt1 has a striking similarity to mechanisms at the dorsoventral boundary in the *Drosophila* wing imaginal disc.

Key words: Hindbrain, Boundary, *wnt1*, Neurogenesis, Lateral inhibition

Introduction

The formation of localised sources of signals that regulate cell differentiation underlies the temporal and spatial patterning of many tissues during embryogenesis. In a number of tissues, this is a component in the progressive refinement of pattern in which following an initial subdivision into distinct regional domains, local interactions induce a signalling centre at the interface of domains. For example, in the wing imaginal disc of *Drosophila*, signalling molecules expressed at the anteroposterior and dorsoventral compartment boundaries (Dpp and Wg, respectively) have a crucial role in long-range patterning of the tissue (Irvine and Rauskolb, 2001). An analogous situation occurs in the vertebrate neural epithelium in which a signalling centre expressing Fgf8 and Wnt1, the isthmic organiser, forms at the interface of the midbrain and hindbrain (Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). In order for such localised signals to correctly pattern the adjacent tissue, it is essential that the interface at which the signalling source forms is sharp and straight, and that the location and number of signalling cells are precisely regulated (Dahmann and Basler, 1999). Important insights into underlying mechanisms have come from studies in *Drosophila* that have revealed receptor-ligand systems that regulate the restriction of cell intermingling, and that control the formation of signalling centres at boundaries (Irvine and Rauskolb, 2001).

In vertebrates, the segmentation of the hindbrain to form rhombomeres provides an amenable model for studying regional specification and the formation of precise patterns of cell differentiation. Each rhombomere has a distinct anteroposterior identity regulated by *Hox* gene expression that underlies the generation of segmentally organised neurons (Lumsden and Krumlauf, 1996). The sharp and straight interface between each rhombomere is stabilised by the restriction of cell intermingling by Eph receptor-ephrin signalling (Fraser et al., 1990; Mellitzer et al., 1999; Xu et al., 1999). Interactions between adjacent rhombomeres induce the formation of boundary cells at the segment interfaces that have a distinct morphology from non-boundary cells and express a number of specific molecular markers (Guthrie et al., 1991; Guthrie and Lumsden, 1991; Heyman et al., 1995; Heyman et al., 1993; Lumsden and Keynes, 1989; Xu et al., 1995). Because boundary cells have reduced cell proliferation and interkinetic nuclear migration compared with other neural epithelial cells (Guthrie et al., 1991), they may act as a non-motile cell population that stabilises the interface of segments. A further potential role of boundaries is suggested by studies of cell organisation in the zebrafish hindbrain that reveal an anteroposterior organisation of neuronal and glial cell types

within each rhombomere (Hanneman et al., 1988; Metcalfe et al., 1986; Trevarrow et al., 1990). For example, primary reticulospinal neurons are located at the centre of rhombomeres, and a GFAP-expressing glial cell 'curtain' forms adjacent to segment boundaries (Trevarrow et al., 1990). The formation of this stereotypical pattern can most easily be explained by a role of segment boundaries as signalling centres that regulate the positioning of cell differentiation.

In recent work, we found that expression of *radical fringe* (*rfng*), a modulator of Notch signalling, occurs in boundary cells in the zebrafish hindbrain, and that Delta ligands are expressed in non-boundary regions (Cheng et al., 2004; Qiu et al., 2004). Activation of Notch receptor in boundary cells regulates their affinity such that they remain segregated to segment interfaces, and mediates lateral inhibition that prevents the premature neurogenesis of boundary cells (Cheng et al., 2004). *Rfng* function is required to upregulate *wnt1* expression in hindbrain boundary cells but not in the roof plate (Cheng et al., 2004). To identify molecular mechanisms that may mediate potential roles of hindbrain boundaries in patterning, we set out to analyse functions of the Wnt1 signalling molecule. We show that Wnt1 regulates proneural and *delta* gene expression in non-boundary regions in the zebrafish hindbrain, and that this mediates lateral inhibition that prevents the spreading of hindbrain boundaries. Wnt1 acts in a regulatory network that is strikingly similar to that occurring at the dorsoventral compartment boundary in the *Drosophila* wing imaginal disc.

Materials and methods

Fish maintenance

Wild-type zebrafish embryos were obtained by natural spawning and raised at 28°C, as described (Westerfield, 1994). Control and injected embryos were stage-matched based on morphology (number of somites, pigmentation of eye) (Kimmel et al., 1995).

Morpholino oligonucleotide and RNA injections

Morpholino oligonucleotides (MO) were purchased from Gene Tools (Oregon, USA). One- to four-cell blastomeres were microinjected with 0.5-2.5 pmol of MO. 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 previously (Cheng et al., 2004); and
tcf3b MO, as described previously (Dorsky et al., 2003).

Capped RNAs encoding full-length chick Wnt1 (cWnt1) and stabilised β-catenin (β-cat), lacking 87 amino acids that include the phosphorylation site for GSK3 (Domingos et al., 2001), were synthesised as described previously (Xu et al., 1995). To achieve mosaic expression, 0.2-1.0 ng of RNA was injected into one cell at the two- to eight-cell stage. In the case of embryos injected with both *wnt1* MO and 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.

In situ hybridisation and immunohistochemistry

In situ hybridisation probes have previously been described as follows: *rfng* (Cheng et al., 2004; Qiu et al., 2004), *foxb1.2* (Moens et al., 1996), *wnt1* (Molven et al., 1991), *asha* and *ashb* (Allende and Weinberg, 1994), *ngn1* (Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998), *p27^{xic1}-a* (Geling et al., 2003), *cyclind1* (Yarden et al., 1995), *deltaA* and *deltaD* (Haddon et al., 1998), *dbx1a* (Fjose et al., 1994), *tbx20* (Ahn et al., 2000), and *gfap* (Nielsen and Jorgensen, 2003). Digoxigenin-UTP labeled riboprobes were synthesized according to the manufacturer's instructions (Roche) and in situ hybridisation was performed as described previously (Xu et al., 1994). The colour reaction was carried out using NBT/BCIP substrate (Roche). Embryos were then re-fixed in paraformaldehyde and mounted for photography on a Zeiss Axiovision microscope, or processed for immunohistochemistry.

Primary antibodies used are as follows: anti-EphA4 [1:500 (Irving et al., 1996)], anti-HuC/HuD (1:200, Molecular Probes), anti-phosphohistone H3 (1:250, Upstate) and anti-neurofilament (RMO-44, 1:200, Zymed). Antibodies were diluted in PBS-Tween containing 2% goat serum, and embryos were blocked in 5% goat serum. Detection of primary antibodies was carried out using Alexa Fluor-488, -594 or -647 goat anti-mouse or anti-rabbit IgG (1:500, Molecular Probes), or HRP-conjugated goat anti-rabbit IgG (1:250, Dako). Fluorescent images were captured using a Leica TCS SP2 confocal microscope.

Results

Expression of *wnt1* in hindbrain boundaries

Previous studies have revealed important roles of *wnt1* at the midbrain/hindbrain organiser (Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001) and roof plate (Megason and McMahon, 2002). We find that in addition to being detected at these sites, *wnt1* transcripts are present in hindbrain boundary cells in zebrafish embryos. *wnt1* transcripts are first detected in hindbrain boundaries from 14 hours of development (not shown), and at 16.5 and 18 hours are present in the dorsal half of all boundaries (Fig. 1A-C). At these stages, *wnt1* expression occurs in the roof plate of rhombomeres r2, r3 and r5 (Fig. 1C). By 24 hours, *wnt1* expression in the hindbrain occurs in the dorsal roof plate of all segments, except r1, and continues to occur in the dorsal part of hindbrain boundaries (Fig. 1D). We compared the expression of *wnt1* with *rfng* and *foxb1.2* (*mariposa*) that are expressed along the whole dorsoventral extent of hindbrain boundaries. Expression of *rfng* in two rows of boundary cells that flank the interface of segments (Cheng et al., 2004; Qiu et al., 2004) is maintained throughout the period of *wnt1* expression (Fig. 1E-H). *foxb1.2* is expressed at low levels in non-boundary regions, and upregulated at hindbrain boundaries (Cheng et al., 2004; Moens et al., 1996) (Fig. 1I-L). As upregulation of *foxb1.2* expression at boundaries does not require rfng function, or vice-versa (Fig. 2J, and data not shown), these genes are independent markers of boundary cells.

Effect of *wnt* pathway knockdowns on hindbrain boundaries

We examined whether *wnt1* has a role in the formation of hindbrain boundaries by using an antisense morpholino oligonucleotide (MO) to block translation of *wnt1* mRNA, and then assessed boundary marker expression. We find that at 18 hours, *rfng* expression appears unchanged in *wnt1* MO-injected embryos compared with uninjected embryos (Fig. 2A,B). By contrast, only 1.5 hours later at 19.5 hours of development, *rfng* expression is broader at all boundaries in *wnt1* MO embryos, and in some embryos extends to the centre of the rhombomeres (Fig. 2C,D). A similar broadening of *rfng* expression (Fig. 2E,F) and of *foxb1.2* boundary expression (Fig. 2G,H) is

observed in *wnt1* MO embryos at 26 hours. Notably, ectopic boundary marker expression does not occur in rhombomere 4 (r4; Fig. 2D,F). Ectopic boundary marker expression does not occur following injection of control morpholino with scrambled sequence (data not shown).

In recent work, we have shown that modulation of Notch activation by Rfng is required for expression of *wnt1* at hindbrain boundaries (Cheng et al., 2004). As roof plate expression of *wnt1* is unaffected by rfng knockdown, this allowed us to assess the role of the boundary expression of *wnt1*. We find that in *rfng* MO embryos, the boundary expression domains of *rfng* and of *foxb1.2* expand (Fig. 2I,J), as happens in *wnt1* MO embryos. Taken together with the *wnt1* MO experiments, these findings suggest that the boundary expression of *wnt1* is required to prevent boundary marker expression from broadening. It is possible that *rfng* is also required for the expression of other boundary signals that contribute to the restriction of boundary spreading.

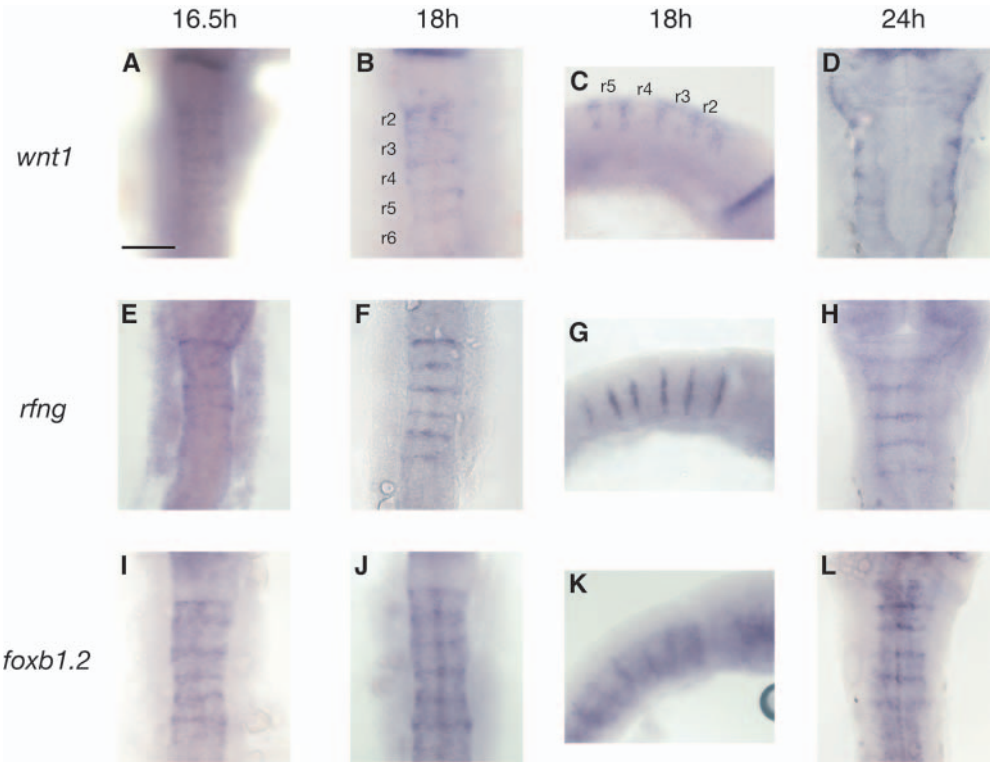

Fig. 1. Expression of *wnt1* and other markers at hindbrain boundaries. *wnt1* (A-D), *rfng* (E-H) and *foxb1.2* (I-L) transcripts are detected in rhombomere (r) boundaries at the indicated stages. Lateral views (C,G,K) reveal that *wnt1* expression is restricted to the dorsal half of boundaries at 18 hours. *wnt1* transcripts are also detected in the roof plate (A-D). *rfng* expression is detected only in boundary cells in the hindbrain, whereas *foxb1.2* transcripts are also present at lower levels within the rhombomere centres. C, G and K are lateral views, anterior at the right; other panels are dorsal views, anterior at the top. Scale bar: 100 μm.

Previous studies have suggested that *tcf3b*, a mediator of canonical Wnt signalling, is required for the formation of hindbrain boundaries, as *tcf3b* MO-injected zebrafish embryos lacked morphological hindbrain boundaries, and *foxb1.2* expression at boundaries appeared to be absent (Dorsky et al., 2003). However, the uniform expression of *foxb1.2* observed in this study could be due to ectopic hindbrain boundary expression, rather than an absence of boundary expression and persistence of the non-boundary expression of *foxb1.2*. We therefore examined the role of *tcf3b* in more detail. Knockdown of *tcf3b* causes an expansion of the expression domains of both *rfng* and *foxb1.2* (Fig. 2K,L), as occurs in *wnt1* knockdowns. Our observation of the same phenotype following knockdown of two distinct genes in the same pathway is strong evidence for the specificity of this effect of the morpholino oligonucleotides, and suggests that the action of Wnt1 in the hindbrain is mediated by Tcf3b.

There are several possible explanations for the expansion of boundary marker expression following knockdown of *wnt1* or *tcf3b*. Because hindbrain boundary markers are upregulated by interactions between cells with distinct segmental identity at segment interfaces (Guthrie and Lumsden, 1991), the broadening of boundary marker expression could be due to a mixing or interleaving of interfaces. However, we detect no difference from uninjected embryos in the formation of sharp segmental expression domains of *hoxb1a* and *krox20* in *wnt1* MO embryos in which ectopic boundary marker expression has occurred (data not shown). An alternative possibility is that decreased *wnt1* function leads to a major increase in boundary cell proliferation and loss of non-boundary cells during the period of boundary marker expression expansion. To examine this, we detected phospho-histone H3, a marker of mitosis, and found that during boundary marker expansion at 19 hours there is a decrease in mitotic index (Fig. 2M,N,Q), both of boundary cells (42% decrease) and of non-boundary cells (30% decrease). Furthermore, we find that cyclinD1, a known downstream transcriptional target of Wnt1 signalling associated with cell proliferation in the spinal cord (Megason and McMahon, 2002), is expressed at elevated levels in hindbrain boundaries (Fig. 2O). In *wnt1* MO embryos, cyclinD1 expression at boundaries is decreased (Fig. 2P), suggestive of a role of Wnt1 in enabling rather than constraining boundary cell proliferation. We therefore favour a third explanation for the broader expression domains of hindbrain boundary markers: that Wnt1 signalling via Tcf3b is required to prevent non-boundary cells from upregulating boundary markers.

Expression of proneural and *delta* genes in the hindbrain

A potential mechanism of Wnt1 action was suggested by the expression patterns of *achaete-scute* homologue (*ash*) and neurogenin (*ngn*) proneural genes, and of Notch ligands in the

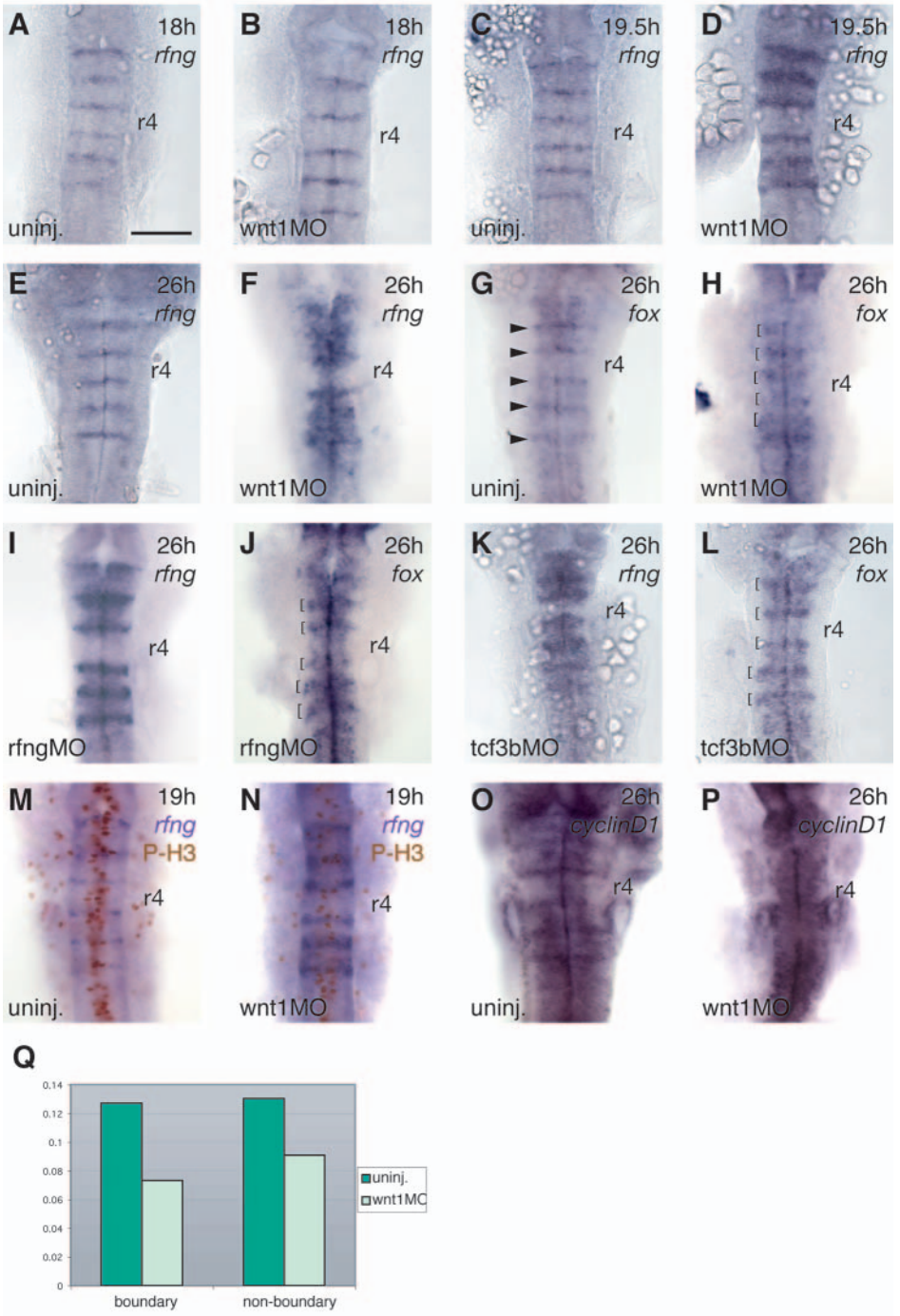

Fig. 2. Expansion of boundary marker gene expression domains in Wnt pathway knockdowns. Markers are indicated at the top right of each panel. (A-F) Time-course of the effect of *wnt1* morpholino (MO) on *rfng* expression. No difference between uninjected (uninj) and *wnt1* MO embryos is detected at 18 hours (13/14 embryos; A,B), but by 19.5 hours, the *rfng* expression domain is expanded in *wnt1* MO-injected embryos (11/11 embryos; C,D). Expanded *rfng* and *foxb1.2* (*fox*) boundary expression is detected in *wnt1* MO embryos at 26 hours (14/14 embryos; E-H). Arrowheads in G indicate the boundary expression domain of *foxb1.2*; brackets in H indicate expansion of this domain in *wnt1* MO embryos. Ectopic boundary marker expression does not occur in r4. (I-L) Effect of *rfng* (30/38 embryos; I,J) and *tcf3b* (48/54 embryos; K,L) knockdown on expression of boundary markers, as indicated. Brackets in J and L indicate the expanded boundary domain of *foxb1.2* expression (compare with G). (M-Q) Regulation of cell proliferation by Wnt1. (M,N) Double labelling with *rfng* (blue signal) and anti-phosphohistone H3 (brown signal) in uninjected and *wnt1* MO-injected embryos at 19 hours. (O,P) *cyclinD1* is expressed at high levels in hindbrain boundaries at 24 hours in uninjected embryos but not in *wnt1* MO-injected embryos. (Q) Graph of mitotic index in boundary cells (*rfng* expressing) and non-boundary cells in uninjected and *wnt1* MO 19-hour embryos. This was calculated by counting the total number of cells and the number of mitotic cells in the hindbrain of uninjected (n=14 embryos) and *wnt1* MO-injected embryos (n=24). The total cell number in the hindbrain of *wnt1* MO embryos is 84% of that of uninjected embryos. In *wnt1* MO embryos the mitotic index was decreased by 42% in boundary regions and 30% in non-boundary regions. Scale bar: 100 μm.

hindbrain (Cheng et al., 2004). At 18 hours, the proneural genes *asha* and *ngn1* are expressed in cells in the dorsal and ventral half of each rhombomere, respectively, but are excluded from boundaries. At the same stage, *ashb* is expressed at high levels in cells in r4 (Fig. 3A-C). Subsequently, proneural gene expression resolves into stripes that become established by 24 hours and persist until at least 48 hours of development. For example, at 26 hours *ashb* and *ngn1* are expressed in presumptive neuroblasts adjacent to rhombomere boundaries, with weaker expression throughout the ventricular zone except at boundaries (Fig. 3G,H). At this stage, *asha* expression occurs in scattered cells but is excluded from boundaries (Fig. 3F), and from 30 hours is found in stripes adjacent to boundaries (data not shown). Expression of these genes at 24-48 hours is patterned along the dorsoventral axis, with *ngn1* being most ventral, *ashb* medial, and *asha* most dorsal. We found a similar progression in the expression patterns of *delta* genes, which are downstream targets of proneural genes, and of $p27^{Xic1}$-a, which mediates *cdk* inhibition and cell cycle exit in neuroblasts (Carruthers et al., 2003; Geling et al., 2003; Ohnuma et al., 1999). At 18 hours, expression of *deltaA*, *deltaD* and $p27^{Xic1}$-a occurs throughout the rhombomeres, except in boundary cells, with stronger expression seen at lateral locations (Fig. 3D,E; Fig. 4A). By 26 hours, stripes of

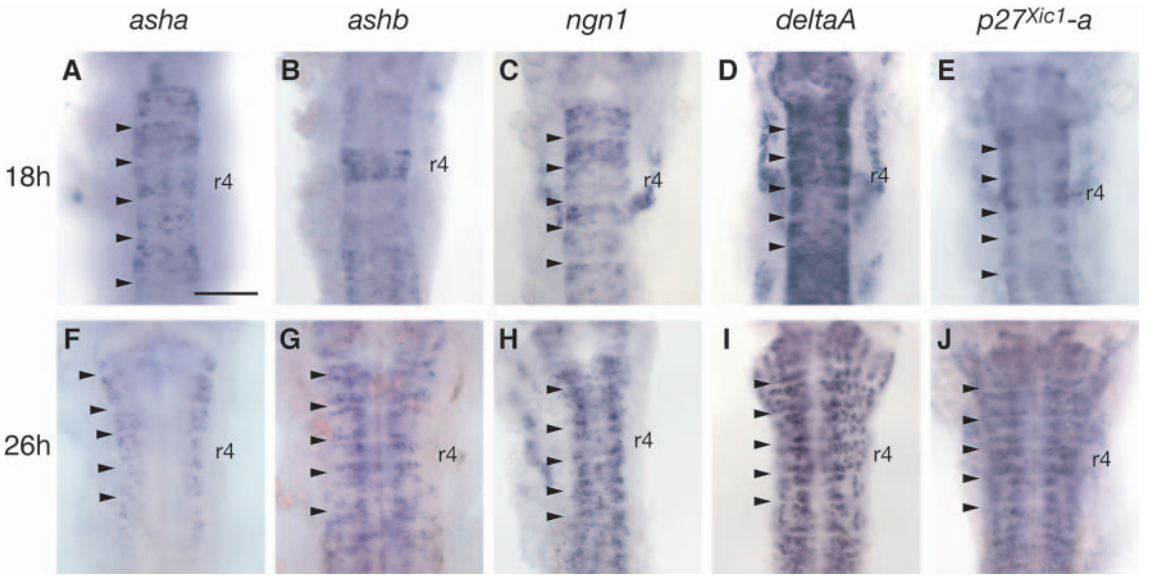

Fig. 3. Patterns of neurogenesis in the zebrafish hindbrain. Expression of *asha* (A,F), *ashb* (B,G), *ngn1* (C,H), *deltaA* (D,I) and *p27^{xic1}-a* (E,J) at 18 hours (A-E) and 26 hours (F-J). At both these stages expression is excluded from boundaries (arrowheads). At 18 hours, transcripts for these genes are detected in all segments; *ashb* is detected at high levels in r4 (B). At 26 hours, stripes of proneural, *deltaA* and *p27^{xic1}-a* gene expression occur adjacent to hindbrain boundaries (*asha* is expressed in stripes adjacent to boundaries by 30 hours). *deltaD* transcripts (not shown) have a similar distribution to those of *deltaA* (D,I). Scale bar: 100 μm.

high level *deltaA, deltaD* and p27^{Xic1}-a expression have formed adjacent to the boundaries, with weaker expression in the ventricular zone in rhombomere centres (Fig. 3I,J and data not shown).

These expression patterns are reminiscent of the situation at the dorsoventral boundary of the *Drosophila* wing disc, where Notch activation at the boundary upregulates Wingless (Wg) expression, which in the anterior compartment signals to neighbouring cells to upregulate *achaete-scute* complex (*as-c*) proneural genes that activate *Delta* expression (Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; Rulifson and Blair, 1995). As Delta cell-autonomously blocks Notch activation, this pathway mediates lateral inhibition that prevents spreading of the *Wg* boundary expression domain (de Celis and Bray, 2000; Rulifson et al., 1996). Our finding that expression of the homologous gene families in the zebrafish hindbrain has the same spatial relationships could provide an explanation for the spreading of boundaries when Wnt1 signalling is blocked. We therefore tested first whether there is an equivalent regulatory hierarchy in the zebrafish hindbrain as in the *Drosophila* wing disc, and second, whether proneural and *delta* genes mediate the lateral inhibition of boundary formation by Wnt1.

Effect of *wnt1* knockdowns on neuronal differentiation

Analysis of *deltaD* gene expression in *wnt1* MO embryos revealed that at 18 hours neurogenesis appears unchanged from uninjected embryos, but by 19.5 hours there is a major reduction in the number of expressing cells (Fig. 4A-D). Similarly, there is a major decrease in the number of cells expressing p27^{Xic1}-a in *wnt1* MO embryos (Fig. 4E,F). These results suggest that Wnt1 is not required for early neurogenesis, but is required for neurogenesis from 19.5 hours onwards. In support of this, we find that there are fewer differentiated neurons (marked by expression of Hu; Fig. 4Q,R) at 25 hours in *wnt1* MO embryos compared with in uninjected embryos. Counting of the number of neural epithelial cells in hindbrain segments at 19 hours reveals that *wnt1* MO embryos have approximately 85% of the cell number of uninjected embryos. Because many precursor cells remain, this decrease does not account for the major deficiency in neurogenesis. The width and morphology of the hindbrain was severely affected in *wnt1* knockdowns at later stages due to the major depletion of the mantle layer of post-mitotic neurons.

To determine whether Wnt1 signalling via Tcf3b is required for generation of all neurons or of specific subsets of neurons after 19.5 hours, we analysed expression of proneural and *delta* genes that mark distinct populations of differentiating cells. We found that by 24 hours, *wnt1* or *tcf3b* knockdown led to a major decrease in the number of cells expressing *deltaA* (Fig. 3I, Fig. 4G,H), *ashb* (Fig. 4I-K), *ngn1* (Fig. 4M-O), *asha* and *deltaD* (not shown) throughout r2, r3, r5 and r6. The greater decrease in neurogenesis following *tcf3b* knockdown compared with *wnt1* knockdown (Fig. 4G,H) may be due to a contribution of other Wnts acting via Tcf3b. It is notable that knockdown of *wnt1* or *tcf3b* consistently had a weaker effect on neurogenesis in the spinal cord, in the hindbrain posterior to r6, and in r4 (Fig. 4F,J-L,N,O). Importantly, we find that decreased proneural and *delta* gene expression occurs following knockdown of *rfng* that leads to loss of *wnt1* expression at hindbrain boundaries (Fig. 4L and data not shown).

To analyse in more detail whether Wnt1 is selectively required for generation of specific neural cell types, we analysed the organisation of the hindbrain at 48 hours, when there is a well-characterised pattern of neuronal and glial cell types (Trevarrow et al., 1990). We found that following *wnt1* knockdown, generation of primary reticulospinal neurons

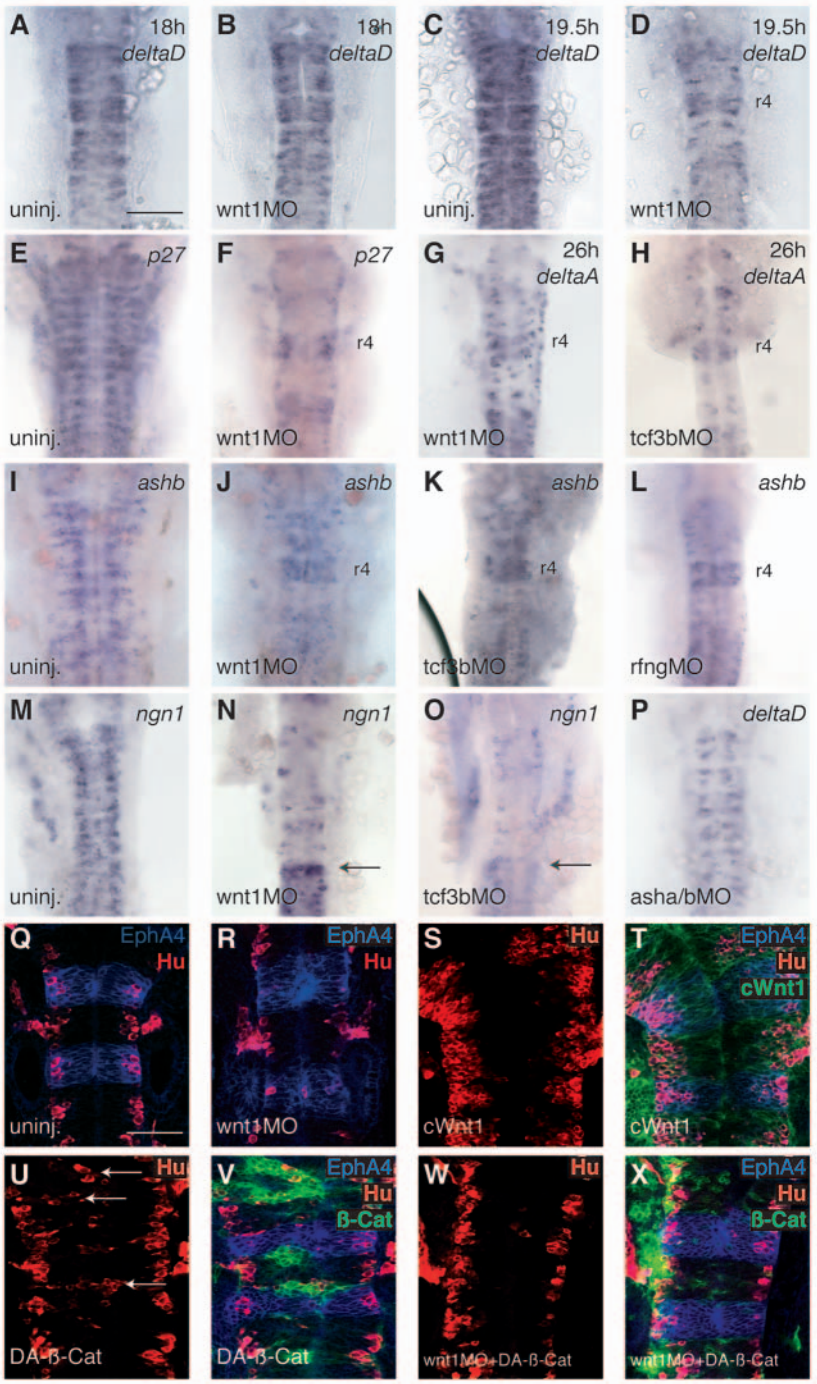

Fig. 4. Regulation of neurogenesis in the hindbrain by Wnt signalling. In situ hybridisation or immunocytochemistry was carried out to detect expression of probe/antigen, as indicated at the top right of each panel. (A-D) Time-course of the effect of *wnt1* knockdown on *deltaD* expression; no effect is observed at 18 hours, whereas expression is much reduced at 19.5 hours (8/10 embryos). (E,F) Neurogenesis is reduced in *wnt1* MO-injected embryos at 24 hours, as seen by detecting $p27^{xic1}$-*a* (*p27*) expression (20/20 embryos). (G-P) Knockdown of *wnt1* (82/86 embryos), *tcf3b* (50/55 embryos) and *rfng* (28/37 embryos) all cause downregulation of delta and proneural gene expression at 24 hours. A greater decrease in *deltaA* expression occurs following knockdown of *tcf3b* than *wnt1* (compare G and H). Notably, neurogenesis is less affected by these knockdowns in r4 (F-H,J-L,N,O), r7 and the spinal cord (arrows in N and O indicate the r6/r7 boundary). (P) Double *asha*/*ashb* knockdown decreases *deltaD* expression. Note that in knockdowns that disrupt neurogenesis, the hindbrain does not broaden and appears to be at an earlier morphological stage due to decreased cell proliferation and major depletion of the mantle zone of postmitotic neurons. (Q-X) Activating Wnt signalling causes ectopic neurogenesis. Hu expression in uninjected (Q), *wnt* MO injected (R), cWnt1 injected (S,T), stabilised β-catenin injected (U,V) or double *wnt* MO and β-catenin injected embryos (W,X). In T, V and X, expressing cells are green (co-injection of GFP), and r3 and r5 are blue (EphA4). Embryos overexpressing Wnt1 have more neurons than uninjected embryos (compare Q and S). β-Catenin can cause ectopic neuronal differentiation in expressing cells in uninjected embryos (arrows in U point to ectopic Hu-positive cells that express β-catenin in V), and in *wnt1* MO embryos (compare left and right sides in W; more neurons are present on the left, where most β-catenin expressing cells are present, X). Scale bars: in A, 100 μm for A-P; in Q, 50 μm for Q-X.

located at rhombomere centres is unaffected (Fig. 5A,B), motor neurons form but in lower numbers (Fig. 5C,D), and there is a major decrease in the number of *dbx1a*-expressing neurons adjacent to segment boundaries, whereas *dbx1a* expression still occurs in progenitor cells (Fig. 5E,F,I-L). Furthermore, *wnt1* knockdown leads to ectopic formation of GFAP-expressing glial cells that in uninjected embryos are located adjacent to hindbrain boundaries (Fig. 5G,H). This pattern is consistent with the requirement for Wnt1 only for later neurogenesis (subsequent to 18 hours of development) revealed by analysis of *delta* gene expression (Fig. 4A-D): most reticulospinal neurons are born prior to 15 hours, motor neurons differentiate between 16-20 hours, and neurogenesis adjacent to hindbrain boundaries occurs between 22-48 hours (Chandrasekhar et al., 1997; Mendelson, 1986; Trevarrow et al., 1990).

Based on previous studies of *delta* gene regulation (Haenlin et al., 1994; Hans and Campos-Ortega, 2002; Heitzler et al., 1996), it seemed likely that the reduction of *delta* gene expression in *wnt1* MO and *tcf3b* MO embryos is due to the decreased expression of proneural genes. Indeed, in either single or multiple knockdowns of *asha*, *ashb* and *ngn1*, we found that *deltaA* and *deltaD* expression is strongly reduced (compare Fig. 4P with Fig. 3I; data not shown).

Effect of Wnt pathway activation on neurogenesis

Gain-of-function experiments suggest that in the mouse spinal cord activation of the Wnt/β-catenin pathway promotes proliferation and inhibits differentiation of neuronal progenitors (Megason and McMahon, 2002; Zechner et al., 2003). We therefore further tested whether Wnt1 promotes neurogenesis in the hindbrain by analysis of the effects of ectopic expression of Wnt1 or stabilised β-catenin (lacking

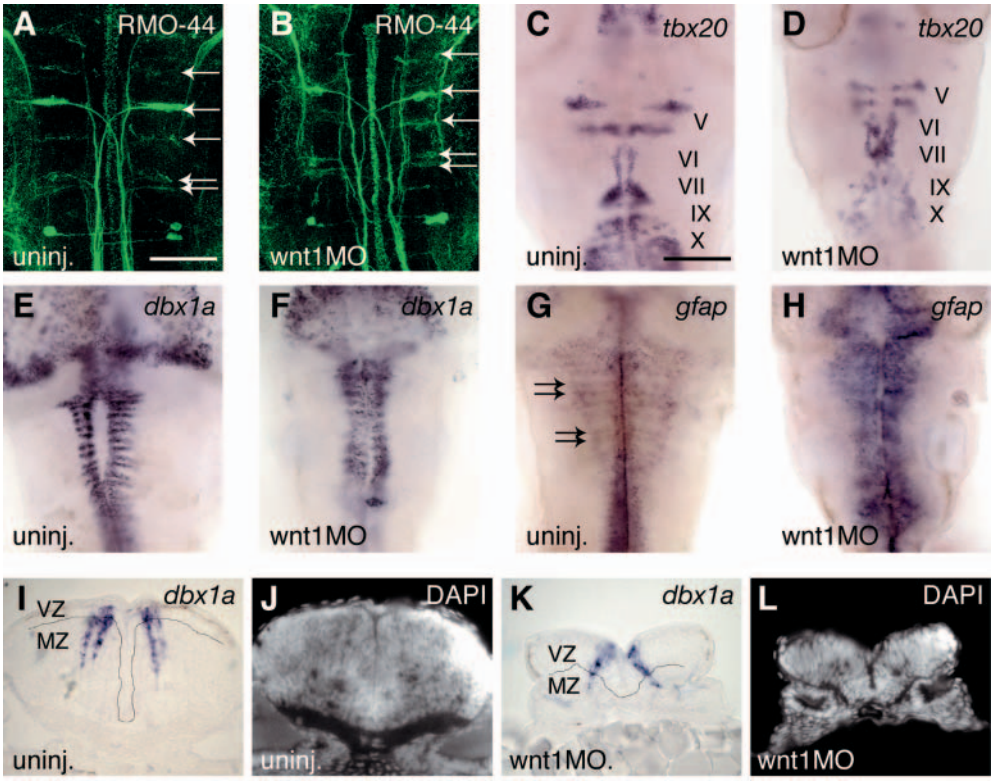

Fig. 5. Neurogenesis at 48 hours in *wnt* MO-injected embryos. (A,B) Reticulospinal neurons labelled by RMO-44 antibody (arrows) are still present (11/14 embryos), and cranial motor nerves (V, VI, VII, IX and X) labelled with *tbx20* (C,D) are mildly hypomorphic (11/11 embryos). The number of *dbx1a*-expressing cells appears to be similar in uninjected and morphant embryos (E,F), but their distribution in stripes adjacent to boundaries is disrupted (20/20 embryos). Sections of uninjected (I,J) and wnt1MO embryos (K,L) show that in uninjected embryos there are many neurons expressing *dbx1a* in the mantle zone (MZ); in the morphants, most *dbx1a* expression occurs in the ventricular zone (VZ) of progenitors (4/4 embryos), as determined by DAPI stain (J,L) and by absence of Hu staining (not shown). (G,H) *gfap* expression occurs in stripes adjacent to boundaries in uninjected embryos (arrows in G), and ectopically in *wnt1* MO embryos (H, 12/13 embryos), suggesting that neural progenitors have switched fate to glial cells. Scale bar: in A, 50 μm for A,B,I-L; in C, 100 μm for C-H.

sequences required for GSK3-induced degradation). We found that overexpression of Wnt1 (Fig. 4S,T) or of stabilised β-catenin (Fig. 4U,V) led to an increase in the number of differentiated neurons in comparison to uninjected control embryos (Fig. 4Q). Furthermore, expression of stabilised β-catenin in *wnt1* MO embryos (Fig. 4W,X) rescues the decrease in neurogenesis in embryos injected with *wnt1* MO (Fig. 4R). These findings suggest that Wnt1 promotes rather than inhibits neuronal differentiation in the hindbrain.

Role of proneural and *delta* genes in repressing boundary markers

We next analysed whether, analogous to the situation in the *Drosophila* wing disc, proneural and *delta* gene function is required to repress ectopic boundary marker expression. We find that expansion of *rfng* and *foxb1.2* expression occurs in *asha* MO embryos, to a lesser extent in *ashb* MO injected embryos, and with a stronger phenotype in *asha/ashb* double knockdowns (Fig. 6A-D,I-K). Knockdown of *ngn1* does not lead to hindbrain boundary spreading, but synergises in a double knockdown with *ashb* (Fig. 6E,F). These synergistic effects suggest that proneural genes overlap in restricting boundary spreading. As found in *wnt1* and *tcf3b* knockdowns, boundary spreading does not occur in r4 in multiple knockdowns of proneural genes. Remarkably this was the case even following triple knockdown of *asha*, *ashb* and *ngn1*, which almost completely blocks neurogenesis (Fig. 6G). Finally, we analysed the effect of decreased *delta* gene function. Hindbrain boundary expansion is not detected in homozygous *after eight* mutant embryos that have a null mutation in the deltaD gene (data not shown). However, knockdown of *deltaA* leads to a major expansion of *rfng* and *foxb1.2* boundary expression (Fig. 6H,L).

Discussion

Previous work has shown that a distinct population of boundary cells forms at the interface of odd and even hindbrain segments (Guthrie and Lumsden, 1991; Lumsden and Keynes, 1989), but little is known regarding the functions of these cells or mechanisms that regulate their formation. One potential role of boundary cells is to inhibit mixing across the interfaces of segments (Guthrie et al., 1991), and consistent with this recent work has identified a role of Notch signalling in regulating an affinity difference between boundary cells and non-boundary cells (Cheng et al., 2004). Here, we have found that expression of Wnt1 at hindbrain boundaries regulates neurogenesis and prevents the spreading of boundaries. Hindbrain boundaries therefore have an essential role in the control of cell differentiation in the zebrafish hindbrain.

Roles of *wnt1* in proliferation and neurogenesis

A key question is the nature of the relationship between Wnt1 and the control of neurogenesis in the zebrafish hindbrain. A number of studies have found that activation of the Wnt/β-catenin pathway promotes neural precursor proliferation and inhibits neuronal differentiation (Chenn and Walsh, 2002; Megason and McMahon, 2002; Zechner et al., 2003). For example, Wnt1 expression in the roof plate of the mouse spinal cord acts via upregulation of cyclinD1 to drive cell proliferation, which expands the neuronal precursor population and inhibits neuronal differentiation in dorsal regions

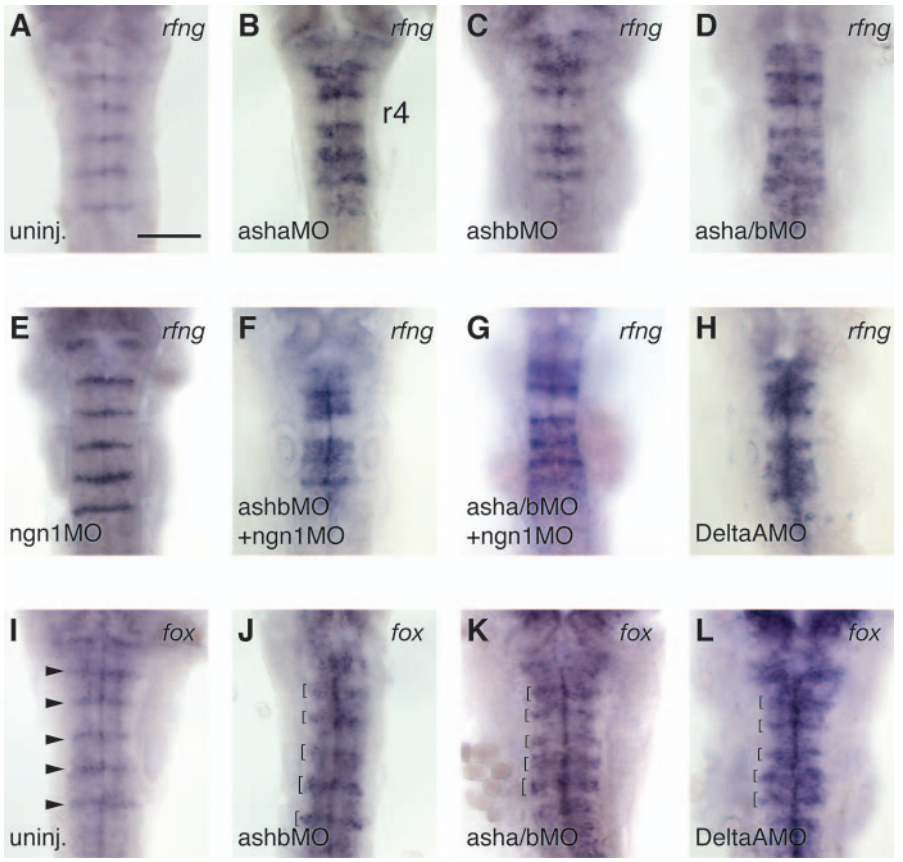

Fig. 6. Repression of boundary markers by proneural genes and *deltaA*. Expression at 24 hours of *rfng* (A-H) and *foxb1.2* (*fox*, I-L) in uninjected embryos (A,I) and embryos injected with morpholinos to block proneural gene or *deltaA* function, as indicated (B-H,J-L). The probe is indicated at the top right of each panel. Expansion of *rfng* and *foxb1.2* boundary expression occurs in proneural gene knockdowns (121/145 embryos), and following *deltaA* knockdown (24/24 embryos). Arrowheads in I indicate boundary expression of *foxb1.2*, which is broader in MO embryos (brackets, J-L). Different strengths of phenotype are observed following proneural gene knockdown, *asha* knockdown being the strongest (B) and *ngn1* the weakest (E). Ectopic expression is not seen in r4, even in a knockdown of *asha*, *ashb* plus *ngn1* (G). Scale bar: 100 μm.

(Megason and McMahon, 2002). Indeed, the observation that neurogenesis occurs first in the centre of zebrafish hindbrain segments (Trevarrow et al., 1990) is suggestive of the situation in the spinal cord in which neurogenesis is initiated distal from the source of *wnt1* expression. However, we find that rather than suppressing neurogenesis, after 18 hours of development *wnt1* is required for neurogenesis in the zebrafish hindbrain.

One possible explanation for our findings is that, as in the spinal cord, *wnt1* promotes cell proliferation, and that *wnt1* knockdown leads to a subsequent lack of neurogenesis in the hindbrain because of a depletion of precursor cells available to differentiate into neurons at 19 hours and later stages. Indeed, *wnt1* knockdown leads to decreased *cyclinD1* expression and cell proliferation in the hindbrain. However, there is only an approximate 15% decrease in the number of neural epithelial cells at 19 hours in *wnt1* knockdowns, with many neural precursors remaining that in r2, r3, r5 and r6 downregulate proneural gene expression and upregulate boundary cell markers. As boundary cells are themselves neural progenitors (with delayed differentiation), the decrease in neurogenesis following *wnt1* knockout is in large part due to a switch of progenitors from non-boundary to boundary identity. In support of an inhibition of neuronal differentiation rather than loss of neural progenitors, we observe that *wnt1* knockdown leads to inhibition of the differentiation of *dbx1a*-expressing neuronal precursors. Furthermore, the ectopic differentiation of GFAP-expressing glial cells in rhombomere centres following *wnt1* knockdown could be due to a block in neuronal differentiation of precursors (Nieto et al., 2001), and/or to the ectopic formation of hindbrain boundary cells. By contrast, proneural gene expression is maintained in the spinal cord, with a sharp anterior boundary at the r6/r7 interface in *wnt1* knockdowns, suggestive of a distinct role of *wnt1* in neurogenesis in the spinal cord compared with in the hindbrain. Intriguingly, the spatial relationship between *wnt1* and *cyclinD1* expression is different in the spinal cord and hindbrain. In the spinal cord, *cyclinD1* is expressed in the dorsal part of neural tube adjacent to the *wnt1*-expressing roof plate, whereas in the hindbrain *cyclinD1* expression occurs at high levels in boundary cells, suggesting that Wnt1 has an autocrine as well as a paracrine role.

We therefore favour a model in which, in addition to a role in regulating cell proliferation, Wnt1 promotes neurogenesis in the hindbrain. Because in *wnt1* or *tcf3b* knockdowns, proneural gene expression is decreased and boundary markers upregulated throughout r2, r3, r5 and r6, Wnt1 seems to act throughout these segments to enable neurogenesis. The expression of *wnt1* in both the roof plate and hindbrain boundaries raises the question of the relative contribution of these signalling sources. We find that loss of the boundary expression but not roof plate expression of *wnt1* in *rfng* knockdowns leads to boundary spreading and decreased neurogenesis. As it is likely that the roof plate expression of Wnt1 also contributes to the promotion of neurogenesis and inhibition of hindbrain boundary spreading, expression by hindbrain boundaries may achieve a threshold level of signal required to drive sufficient proneural gene expression to block boundary spreading.

Our findings could be explained by two alternative roles of Wnt1 signalling. First, that Wnt1 induces a proneural state of non-boundary cells and is a permissive factor for neurogenesis, whereas other factors control the spatial patterns of differentiation. In support of this are the observations that neurogenesis occurs adjacent to the full dorsoventral extent of hindbrain boundaries, whereas *wnt1* expression is restricted to the dorsal part of hindbrain boundaries, and that neurogenesis does not occur in a stripe adjacent to *wnt1* expression in the roof plate of the hindbrain. A second possibility is that in addition

to Wnt1 being required for a proneural cell state through the segment, high levels of Wnt1 induce the differentiation of neurons adjacent to boundaries. This situation is similar to the *Drosophila* wing disc, in which cells adjacent to the source of Wg express proneural genes and, as a consequence, undergo mitotic arrest and differentiate (Couso et al., 1994; Johnston and Edgar, 1998; Phillips and Whittle, 1993). Consistent with this, recent studies in the mouse neocortex and in in vitro cell culture have found that Wnt signalling promotes neurogenesis and directly regulates neurogenin 1 gene expression (Hirabayashi et al., 2004; Israsena et al., 2004; Otero et al., 2004). Furthermore, Wnt signalling may have stage-specific effects, in which it initially promotes proliferation and later induces neuronal differentiation (Hirabayashi et al., 2004). However, the spatial pattern of neurogenesis in the hindbrain cannot be explained based solely on Wnt1 signalling; for example, since neurogenesis adjacent to boundaries persists as *wnt1* expression becomes increasingly dorsally restricted in boundaries. Therefore, other factors are required to cooperate with or overlap functionally with any instructive role of Wnt1 in spatial patterning of neuronal differentiation.

During completion of this work, another study has analysed roles of Wnt signals in the hindbrain (Riley et al., 2004). There are significant differences in the conclusions of these authors compared with the current work. First, it is proposed that Wnt signals from hindbrain boundaries have a purely organising role in the anteroposterior pattern of neurogenesis, whereas we demonstrate a temporal requirement for Wnt signals in neurogenesis that underlies a selective loss of later neurons that differentiate adjacent to boundaries. The data of these authors are consistent with a loss rather than a spatial disorganisation of neurogenesis following knockdown of *wnt* genes or of *tcf3b*. Second, Riley et al. (Riley et al., 2004) propose that Wnt signals are required to maintain hindbrain boundaries, whereas we show that they are required to prevent spreading of boundaries. This reflects that these authors did not have appropriate markers available to identify ectopic boundary cells, although, in agreement with our findings, GFAP-expressing glial cells that normally flank boundaries were observed in ectopic locations following decreased Wnt activity. A third difference is that Riley et al. (Riley et al., 2004) observe only subtle phenotypic changes in a deletion mutant that removes *wnt1* and *wnt10b*, but knockdown of further *wnt* genes (or of *tcf3b*) leads to a similar effect on neurogenesis as we observe following knockdown of *wnt1*. The reason for the quantitative difference is unclear, but might be due to the large deletion that removes *wnt1* and *wnt10b* also removing a gene(s) that has a compensatory effect on neurogenesis.

A conserved pathway of neural differentiation and lateral inhibition

A potential pathway by which Wnt1 might inhibit ectopic expression of boundary cell markers was suggested by the similarity of gene expression patterns in the zebrafish hindbrain to those adjacent to the dorsoventral boundary of the *Drosophila* wing imaginal disc. In the wing imaginal disc, expression of *Fringe* and *Serrate* in the dorsal compartment, and of *Delta* in the ventral compartment, leads to a stripe of Notch activation at the dorsoventral boundary (Panin et al., 1997), and Notch activation upregulates *wg* expression (Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; Rulifson and Blair, 1995). Wg

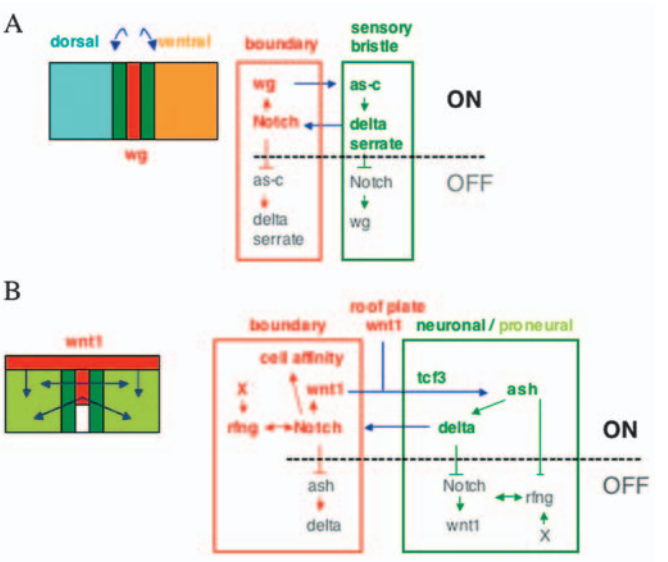

Fig. 7. Model of regulation of cell differentiation and restriction of boundary spreading. The diagrams illustrate the similar regulatory hierarchy of gene regulation and intercellular signalling in (A) the anterior compartment of the *Drosophila* wing disc and (B) the zebrafish hindbrain. In both systems, localised expression of *wg/wnt1* is induced by fringe-mediated modulation of Notch activity at boundaries. In the hindbrain, there is in addition fringe-independent expression of Wnt1 in the roof plate. Wg/Wnt1 acts on non-boundary cells to upregulate proneural gene expression (*as-c/ash*) and thus induce or enable a neural fate. In the wing disc, proneural expression only occurs in neighbouring cells, whereas in the zebrafish hindbrain Wnt1 acts at longer range to promote neurogenesis throughout the segments. Proneural genes upregulate Delta expression, which in turn activates Notch in boundary cells. In addition, proneural gene products and/or Delta cell autonomously suppress boundary cell formation, thus preventing spreading of boundaries. Because in the zebrafish, Notch activation is not sufficient to induce boundary cell marker expression (Cheng et al., 2004), another factor (X) is proposed to be required for boundary cell specification.

protein acts on adjacent cells in the anterior compartment to upregulate expression of *as-c* proneural genes, which specify a post-mitotic sensory hair cell fate, and upregulate Delta gene expression (Couso et al., 1994; Johnston and Edgar, 1998; Phillips and Whittle, 1993). Delta acts cell autonomously to inhibit Notch activation, and because Notch activation is required to activate *wg* expression, this mediates a lateral inhibition that prevents spreading of the *wg* expression domain (de Celis and Bray, 2000; Rulifson et al., 1996) (Fig. 7A).

After 18 hours of development, expression of *ash* and *ngn* proneural genes, and of *delta* genes, becomes restricted to stripes adjacent to hindbrain boundaries. We find that there is the same regulatory hierarchy in the hindbrain (Fig. 7B) as in the *Drosophila* wing disc: the modulation of Notch by Rfng upregulates *wnt1* in boundary cells (Cheng et al., 2004), knockdown of *wnt1* or *tcf3b* leads to a major decrease in the number of cells expressing the *ash* and *ngn* proneural genes, and knockdown of *ash* or *ngn* leads to a decrease in *delta* gene expression. Finally, we find that knockdown of *ash*, *ngn* or *delta* gene function leads to spreading of hindbrain boundary marker expression.

We have thus uncovered a striking parallel with the

mechanisms that in the *Drosophila* wing disc control formation of sensory hair cells and prevent ectopic boundary cell formation. As the zebrafish hindbrain and *Drosophila* wing are not homologous structures, we propose that this reflects an independent recruitment during evolution of a regulatory network of genes that underlies an activity of boundaries both in patterning adjacent tissue and in preventing the spread of the signalling source. There are several differences in the Notch-Wnt-proneural network between the hindbrain and wing imaginal disc; for example, in the expression patterns of *fng* and Notch ligands that establish a stripe of Notch activation at boundaries. This may be due to the larger number of *fng* genes in vertebrates that have distinct expression domains in hindbrain segments and boundaries (Cheng et al., 2004; Prince et al., 2001), and may have diverged in their function (Dale et al., 2003). Another difference is that in the hindbrain, Wnt1 has a long-range role in promotion of neurogenesis, whereas in the wing imaginal disc, neural cells only form immediately adjacent to the Wg signalling source.

A model of boundary and proneural patterning in the hindbrain

Taken together with previous work, our findings reveal a regulatory loop between boundary cells and non-boundary regions that stabilises the pattern of each cell population via bidirectional lateral inhibition (Fig. 7B). Rfng-mediated modulation of Notch activation upregulates *wnt1* expression in boundary cells (Cheng et al., 2004). Notch activation also regulates the affinity properties of boundary cells (Cheng et al., 2004), thus maintaining their segregation to the interfaces of segments. Wnt1 expressed by boundary cells promotes proneural and *delta* gene expression in non-boundary regions, which enables neuronal differentiation and laterally inhibits the spread of boundary marker expression. In addition, roof-plate expression of Wnt1, which is independent of Rfng function, contributes to the promotion of neurogenesis, but is not sufficient to prevent hindbrain boundary spreading. Delta expression by non-boundary cells activates Notch in boundary cells, and thus laterally inhibits boundary cells from expressing proneural genes and undergoing neuronal differentiation (Cheng et al., 2004).

As *wnt1* knockdown affects neurogenesis only after 18 hours of development, this model predicts that other signals from boundaries regulate neurogenesis and restrict boundary spreading at earlier stages of differentiation in the hindbrain. In addition, it is striking that specifically in r4 there is less downregulation of proneural gene expression following *wnt1* or *tcf3b* knockdown, suggesting that other factors regulate neurogenesis in r4. This continued proneural gene expression in r4 could provide an explanation for why there is no boundary spreading into r4 following *wnt1* or *tcf3b* knockdown. However, we find that even following an almost complete block of neurogenesis in a triple knockdown of proneural genes, hindbrain boundary spreading did not occur into r4. The mechanism underlying the restriction of boundary spreading in r4 is not known, but a potential functional significance is suggested by the findings that this rhombomere is the first to form and differentiate, and acts as an early signalling centre to pattern adjacent segments (Maves et al., 2002). This early role of r4 may require that other signals regulate the restriction of boundary spreading and neurogenesis independently of the later upregulation of *wnt1* gene expression in boundaries.

Finally, our findings have implications for mechanisms by which boundary cells form at the interface of adjacent hindbrain segments. An attractively simple model is that boundary cells are specified by interactions that only occur at segment interfaces; for example, by complementary segmental expression of a cell surface receptor/ligand system. However, our finding that after blocking the Wnt1/Tcf3 pathway most or all cells (except in r4) can express boundary markers shows that boundary cell formation does not need to be located adjacent to the segment interface. We therefore propose that interactions at segment interfaces instead provide a bias that ensures that boundary cell specification is initiated at that location, and that lateral inhibition then prevents further cells from becoming boundary cells.

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