

Signalling from hindbrain boundaries regulates neuronal clustering that patterns neurogenesis

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SUMMARY

During central nervous system development, neural progenitors are patterned to form discrete neurogenic and non-neurogenic zones. In the zebrafish hindbrain, neurogenesis is organised by Fgf20a emanating from neurons located at each segment centre that inhibits neuronal differentiation in adjacent progenitors. Here, we have identified a molecular mechanism that clusters *fgf20a*-expressing neurons in segment centres and uncovered a requirement for this positioning in the regulation of neurogenesis. Disruption of hindbrain boundary cell formation alters the organisation of *fgf20a*-expressing neurons, consistent with a role of chemorepulsion from boundaries. The semaphorins *Sema3fb* and *Sema3gb*, which are expressed by boundary cells, and their receptor *Nrp2a* are required for clustering of *fgf20a*-expressing neurons at segment centres. The dispersal of *fgf20a*-expressing neurons that occurs following the disruption of boundaries or of *Sema3fb/Sema3gb* signalling leads to reduced FGF target gene expression in progenitors and an increased number of differentiating neurons. *Sema3* signalling from boundaries thus links hindbrain segmentation to the positioning of *fgf20a*-expressing neurons that regulates neurogenesis.

KEY WORDS: Boundaries, Neurogenesis, Segmentation, FGF signalling, Semaphorin

INTRODUCTION

The complex and stereotyped organisation of neurons in the central nervous system is established during development by the differentiation of progenitor cells in the neural epithelium, their migration to the correct location in the mantle zone, and the extension of axons to connect to appropriate targets. The initial steps require the strict regulation of cell differentiation such that the correct number of each neural cell type is formed while retaining sufficient progenitors to generate later-forming cell types. In part this is achieved through the action of inhibitory factors that limit the amount of differentiation in all progenitors (Bylund et al., 2003; Graham et al., 2003) and through Notch-mediated lateral inhibition within the neural epithelium, in which forming neurons inhibit the differentiation of their neighbours (Louvi and Artavanis-Tsakonas, 2006; Kageyama et al., 2007). In some regions of the neural epithelium, there is also a large-scale patterning of neurogenesis to form discrete neurogenic and non-neurogenic zones (Bally-Cuif and Hammerschmidt, 2003; Diez del Corral et al., 2003). For example, three neurogenic zones form along the dorsoventral axis of the spinal cord during primary neurogenesis due to inhibition of differentiation in the intervening regions by specific *Hes/Her* and *Zic* genes (Brewster et al., 1998; Bae et al., 2005). Similarly, a non-neurogenic zone forms at the midbrain-hindbrain boundary due to the inhibitory action of *Hes/Her* family

members (Geling et al., 2003; Geling et al., 2004). In these examples, the expression of transcription factors that inhibit neuronal differentiation appears to be linked to dorsoventral and anteroposterior patterning within the neural epithelium that underlies cell type and regional specification.

Another striking example of spatially restricted neurogenesis occurs within segments in the zebrafish hindbrain, where neuronal differentiation is absent at the boundaries and centre of each segment (rhombomere) and thus becomes confined to zones flanking each segment boundary (Cheng et al., 2004). Furthermore, specific neuronal and glial cell types are organised in a segmentally repeated pattern; for example, primary reticulospinal neurons are located at each segment centre (Hanneman et al., 1988) and the fibres of radial glial cells form a curtain that flanks segment boundary cells (Trevarrow et al., 1990). These observations raise the question of how the patterning of neurogenesis within segments is established. In view of the mirror-image symmetric organisation of neurogenesis around hindbrain boundaries, one possibility is that each boundary serves as a signalling centre that regulates cell differentiation, analogous to the roles of boundaries in the *Drosophila* wing disc (Irvine and Rauskolb, 2001; Tamagnone and Comoglio, 2004; Cavodeassi and Houart, 2012). Indeed, specialised boundary cells form at the interface of hindbrain segments that have distinct molecular and cellular properties (Guthrie and Lumsden, 1991; Heyman et al., 1995; Xu et al., 1995). The absence of neurogenesis at hindbrain boundaries might be due to Notch activation promoted by Rfng, which is expressed by boundary cells (Cheng et al., 2004; Qiu et al., 2004; Qiu et al., 2009). Apparent support for the role of a boundary signal in organising neurogenesis came from the effects of knockdown of Wnt genes expressed in hindbrain boundaries (Riley et al., 2004; Amoyel et al., 2005); however, later work revealed that the altered neurogenesis was due to non-specific activation of the Bcl-caspase pathway, which has a non-apoptotic role in hindbrain boundary cell formation (Gerety and Wilkinson, 2011).

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A different hypothesis for how boundaries could underlie the patterning of hindbrain neurogenesis is suggested by studies that reveal a key role of *fgf20a*-expressing neurons, which are located in the mantle region at each segment centre and are required for formation of the non-neurogenic zone in the adjacent neural epithelium (Gonzalez-Quevedo et al., 2010). In *fgf20a* mutant embryos, ectopic neurogenesis occurs throughout segment centres, probably owing to a lack of inhibition of neuronal differentiation rather than to altered proliferation of a progenitor population, as FGF signalling does not affect hindbrain cell proliferation at these stages (Gonzalez-Quevedo et al., 2010). Concurrent with the inhibition of neurogenesis, FGF signalling may promote gliogenesis in segment centres (Esain et al., 2010) and thus underlie a switch in cell fate. These findings suggest that it is the localisation of *fgf20a* expression to form a signalling centre in the mantle zone that underlies the stereotyped patterning of neurogenesis. We therefore set out to elucidate how *fgf20a* expression is restricted and, in particular, to address the possibility that it involves signalling from hindbrain boundaries.

By analysing the effects of disrupting hindbrain boundaries, we find that boundary cells are required to maintain a tight cluster of *fgf20a*-expressing neurons in each segment centre. We show that two semaphorin family members expressed at hindbrain boundaries, *Sema3fb* and *Sema3gb*, act through *Nrp2a* to position *fgf20a*-expressing neurons in segment centres. The dispersal of these neurons that occurs upon disruption of semaphorin function leads to an abnormal organisation of neurogenesis and to an increase in the number of differentiating neurons. Hence, there is an interdependence between the stereotyped positioning of *fgf20a*-expressing neurons and the regulation of neurogenesis.

MATERIALS AND METHODS

Fish maintenance and transgenic and mutant zebrafish lines

Zebrafish embryos were obtained by natural spawning and raised at 28.5°C as described (Westerfield, 1993). The *tp53* mutant line (Berghmans et al., 2005) was acquired from the Zebrafish International Resource Center (University of Oregon, Eugene, OR, USA).

Primers

Primers used were (5'-3'): *Sema3fb*Fw(ClaI), ATAATCGATATGCTCT-TGGACAGTCTATGGCCAGTC; *Sema3fb*Re(BsiWI), ATACGTACGT-CATGTCTCTCTCGTCCCG; *Sema3gb*Fw(ClaI), ATAATCGATATG-TCATCTCTGCTTTTCGTCTTG; *Sema3gb*Re(BsiWI), ATACGTACGT-CACTGCTCTCTCTGTTCTCC; *efnb3bEx1*Fw, TTCCGAGTCC-AGAGATCTCCA; *efnb3bEx2*Re, ATGATGAAGTAGTCGTGGTTGG; and *efnb3bIn1*Re, TAATGTGGCGGCTACTGTGC.

Morpholino oligonucleotides

Morpholino oligonucleotides (MOs) were purchased from Gene Tools. One- to four-cell embryos were microinjected with 1.8 nl MO diluted to 5–10 ng/nl in water. All experiments were performed with at least two independent replicates, and injections with control MO were carried out on the same clutch as experimental injections. *tp53*^{-/-} embryos were used for MO injections to avoid off-target gene expression caused by toxicity (Gerety and Wilkinson, 2011). The following MOs (5'-3') have been validated previously: *epha4a* (AACACAAGCGCAGCCATTGGTGTG) (Cooke et al., 2005); *rfng* (TGGAGGCGACATGGGATAAGTGTCAT) (Cheng et al., 2004); *sema3gb* (ATCGCAACATTTCTACCTTTGTAT) (Yu and Moens, 2005); and *sema3fb* (CATAGACTGTCCAAGA-GCATGGTGC), *nrp2a* (CTTGGTGTGATATCCAGAAATCCAT) and *nrp2b* (CGCGTAGAGGAAAAAGCTGAAGTTC) (Tanaka et al., 2007).

Splice-blocking *efnb3b* MO (TTGCGGCTCTTACCTTTTGTTCAG, *efnb3b*-SB MO) was used at 6 ng/nl. A scheme of the effect on transcript production is shown in supplementary material Fig. S1D. The efficacy of this MO was determined by RT-PCR analysis, which showed that *efnb3b* transcript splicing is blocked by *efnb3b*-SB MO but not control MO

(supplementary material Fig. S1F). The loss of *rfng* expression in *efnb3b*-SB MO-injected embryos (supplementary material Fig. S1B) is similar to that observed in embryos injected with translation-blocking *efnb3b* MO (ACTCCCATCAAAGCCGTGTGCGGGA; *efnb3b*-TB) (supplementary material Fig. S1C).

In situ hybridisation and immunohistochemistry

Embryos were grown at 28.5°C to the desired stage, fixed in 4% paraformaldehyde in PBS overnight, then stored in 100% methanol, or processed immediately for in situ hybridisation or immunohistochemistry. The in situ hybridisation probes for *neurog1*, *rfng* (Amoyel et al., 2005), *fgf20a* and *etv5b* (Gonzalez-Quevedo et al., 2010) are as previously described. Based on published sequence information, PCR products were cloned as probe templates into pGemT-Easy (Promega, A1360) using the primers described above for *sema3gb* (AY766121), *sema3fb* (AY766119.1), *nrp2a* (NM_212965.1) and *nrp2b* (NM_212966.1). Digoxigenin-UTP-labelled riboprobes were synthesized according to the manufacturer's instructions (Roche) and in situ hybridisation and colour development with NTB/BCIP were performed as described (Xu and Wilkinson, 1998). Fluorescent in situ hybridisations combined with NTB/BCIP were performed as described (Hauptmann and Gerster, 1994) with minor modifications. Colour detection was performed using Fast Red tablets (Roche, 11496549001).

For antibody staining after fluorescent in situ hybridisation, embryos were washed with PBS containing 0.1% Tween 20, then blocked in PBS containing 0.1% Tween 20 and 5% goat serum. Antibodies were diluted in this blocking solution and incubated overnight at 4°C. Rabbit anti-EphA4 (1:450) (Irving et al., 1996), rabbit anti-GFP (1:200; Torrey Pines, TP 401) and anti-Hu/C/D (1:200; Invitrogen, A21271) were used as primary antibodies and detected with Alexa Fluor 488, 594 or 647 goat anti-rabbit IgG (1:450; Invitrogen). Fluorescent images were captured using a Leica TCS SP2 confocal microscope.

Quantitations

Stacks of confocal images were collected of *fgf20a*-expressing neurons or Hu/C/D-expressing neurons, each combined with EphA4 immunocytochemistry to detect the location of segment interfaces. Images were processed with FIJI (ImageJ) and Adobe Photoshop. To quantitate the distribution of *fgf20a*-expressing neurons, images of r3, r4 and r5 were processed to an identical size along the anteroposterior (AP) axis from one boundary to the next. A scheme of the procedure and examples of clusters in the different MO conditions are shown in supplementary material Fig. S4. The images were used for two quantitations: (1) the AP distance from the rhombomere centre to the furthest edge of the neuron cluster compared with the centre-to-boundary distance (α ; 100 arbitrary units) and (2) the AP distance between the two edges of the cluster compared with the total rhombomere length (β ; 100 arbitrary units).

To quantitate the fusion of r5 and r6 neurons in *efnb3b* morphants, stacks of confocal images were used to measure the AP distance from the posterior edge of the r5 *fgf20a*-expressing neuronal cluster to the anterior edge of the r6 cluster. Stacks of confocal images were used to count the number of Hu-expressing neurons or *fgf20a*-expressing neurons in r3–r5, and to measure the AP length of r3, r4 and r5 in the midline region. All data were processed with Microsoft Excel and the significance of results assessed using the two-tailed Student's *t*-test.

RESULTS

The stereotyped organisation of neurogenesis within hindbrain segments in zebrafish can potentially be explained by a role of hindbrain boundary cells in restricting *fgf20a* expression to neurons (for brevity referred to hereafter as *fgf20a* neurons) located in the centre of segments. Such a role could be mediated through one of two alternative mechanisms: (1) that boundary cells are a source of signals that position neurons which intrinsically express *fgf20a*; or (2) that a diffusible signal emanating from boundary cells extrinsically represses *fgf20a* expression in the vicinity. These models can be assessed by analysing the effects of disrupting the formation of hindbrain boundary cells.

Loss of a subset of segment boundaries leads to repositioning of *fgf20a* neurons

As a first approach toward addressing the potential role of hindbrain boundary cells, we took advantage of the findings that Eph receptor and ephrin signalling not only underlies the formation of sharp segment interfaces, but also is required for the upregulation of boundary cell markers (Xu et al., 1995; Cooke et al., 2005). Knockdown of *epha4a* leads to decreased expression of the boundary cell marker *sema3gb* at some segment interfaces, but not others (Cooke et al., 2005), which is likely to reflect the overlapping expression and function of other Eph receptors. We verified this finding using *rfng* as a boundary cell marker and found loss or substantial downregulation of its expression at the interface between segments 2/3, 3/4 and 5/6, whereas expression was maintained at the 4/5 and 6/7 boundaries (Fig. 1A,B). We found that *epha4a* knockdown leads to an altered organisation of *fgf20a*-expressing cells at 24 hours postfertilisation (hpf): whereas *fgf20a* expression in rhombomere (r) 4 seemed unaffected, the cluster of

fgf20a neurons in r3 was more dispersed, and in r5 and r6 the clusters were relocated and became fused near to the r5/r6 interface (Fig. 1D,E). As the altered organisation is not accompanied by an increase in the number of *fgf20a* neurons (Fig. 1N), these observations argue against boundary cells acting to repress *fgf20a* expression and are more consistent with a role in positioning of *fgf20a* neurons.

An alternative interpretation is that knockdown disrupts the organisation of *fgf20a* neurons in r3 and r5, where *epha4a* is normally expressed, due to a role of this Eph receptor within r3/r5 rather than in boundary cell formation. To address this, we knocked down *ephrin B3b* (*efnb3b*), a ligand of EphA4 (Gale et al., 1996), which is expressed in r2/r4/r6 (Chan et al., 2001), complementary to *epha4a*. We found that *efnb3b* knockdown leads to a similar disruption of boundary marker expression as *epha4a* knockdown (Fig. 1C; supplementary material Fig. S1), consistent with these factors acting as a receptor-ligand pair. By detecting EphA4 protein as a marker of r3/r5 following knockdown of *efnb3b*, we

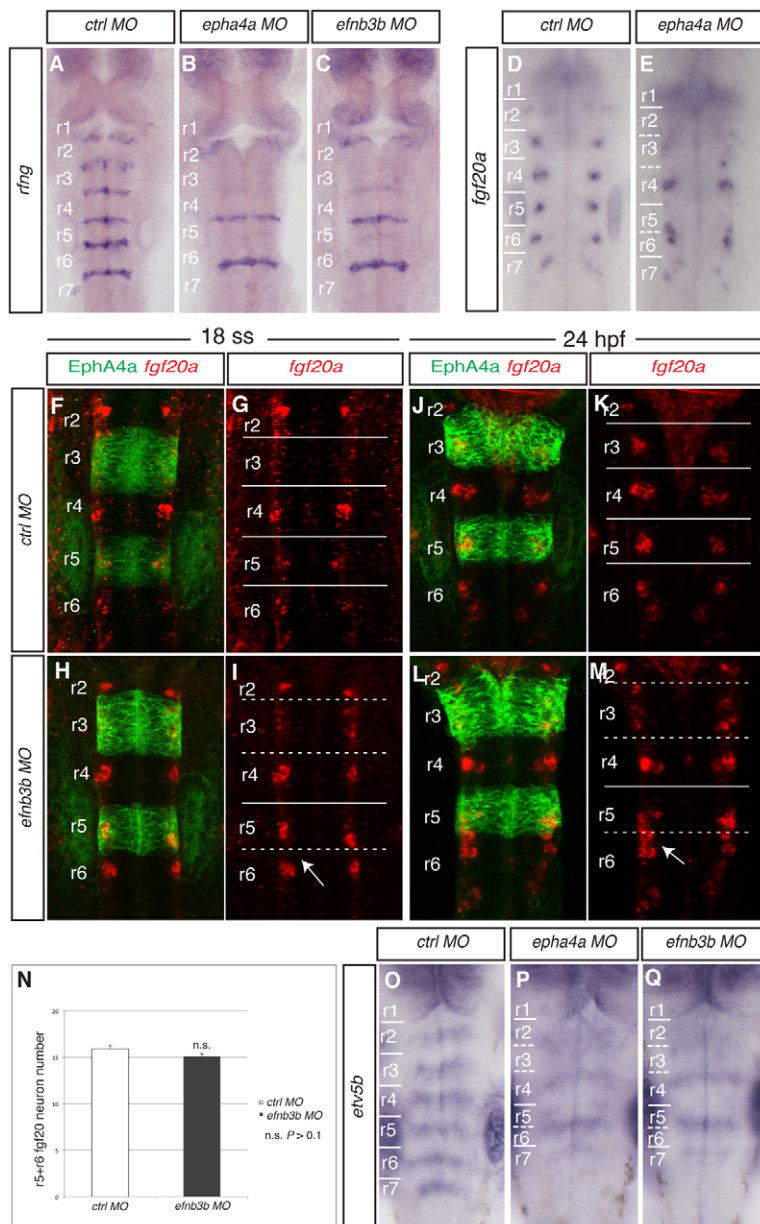


Fig. 1. Disruption of a subset of rhombomere boundaries affects the position of *fgf20a* neurons.

Brightfield and confocal images show dorsal views of the zebrafish hindbrain, anterior to the top, following in situ hybridisation (blue or red) and staining for EphA4 protein (green). (A–C) *rfng* expression in 21 ss embryos after injection of control MO (A), *epha4a* MO (B) or *efnb3b*-SB MO (C). There is decreased boundary marker expression selectively at the r2/r3, r3/r4 and r5/r6 borders following *epha4a* (90%, $n=32$) or *efnb3b* (82%, $n=23$) knockdown. (D,E) *fgf20a* expression at 24 hpf after injection of control MO (D) or *epha4a* MO (E). Knockdown of *epha4a* leads to altered localisation of *fgf20a* neurons (88%, $n=26$). (F–M) *fgf20a* expression (red) and EphA4 antibody staining (green) in embryos injected with control MO (F,G,J,K) or *efnb3b*-SB MO (H,I,L,M) at 18 ss (F–I) and 24 hpf (J–M). Arrow points to r5 and r6 *fgf20a* neuronal clusters that approach and fuse near to the r5/r6 interface. *efnb3b* morphants do not have the r5–r6 fusion of *fgf20a* neurons at 18 ss (95%, $n=20$), but do at 24 hpf (83%, $n=18$). (N) Quantitation of the number of *fgf20a* neurons in r5 plus r6. There is no significant difference between control versus *efnb3b* knockdown embryos (15.9 ± 0.4 versus 15.1 ± 0.3 neurons, average \pm s.e.m., $n=11$ each; $P=0.19$). (O–Q) *etv5b* expression at 30 hpf in embryos injected with control MO (O), *epha4a* MO (P) and *efnb3b*-SB MO (Q). Knockdown of *epha4a* or *efnb3b* leads to an altered pattern with fewer stripes of *etv5b* expression (94%, $n=35$; 88%, $n=24$, respectively). Dashed white lines indicate the position of missing boundary marker expression, and continuous white lines indicate the position of remaining boundary cells. r, rhombomere.

visualised the relationship between *fgf20a* neurons and segment interfaces (Fig. 1F-M). We found that *efnb3b* knockdown leads to a similar alteration in the positioning of *fgf20a* neurons at 24 hpf as *epha4a* knockdown, with a dispersal of *fgf20a* neurons in r3 and an ectopic cluster at the r5/r6 interface (Fig. 1L,M). The r5/r6 clusters are separate at the 18-somite stage (ss) (Fig. 1H,I) but are fused by 24 hpf (Fig. 1L,M), suggesting that they undergo a progressive displacement from their original position at the centre of r5 and r6. The similar effect of knockdown of *epha4a* and *efnb3b* – which in the hindbrain interact only at segment borders – suggests that the altered organisation of *fgf20a* neurons is a consequence of the depletion of boundary cells rather than reflecting an autonomous role in the segments within which they are expressed. The finding that the organisation of *fgf20a* neurons in *efnb3b* morphants appears normal at early stages suggests that boundary cells are not required for the initial positioning of these neurons.

To determine whether the altered position of *fgf20a* neurons affects the pattern of FGF receptor activation, we analysed the expression of *etv5b* (*erm*), a target of Fgf20a signalling at this stage of development (Gonzalez-Quevedo et al., 2010). In control embryos, *etv5b* is restricted to the centre of each of the six rhombomeres (Fig. 1O). Knockdown of either *epha4a* (Fig. 1P) or *efnb3b* (Fig. 1Q) leads to a similar change, in which there are fewer stripes of *etv5b* expression, and these correlate with the altered position of *fgf20a* neurons in these knockdown conditions: there is strong *etv5b* expression in r4 and at the r5/r6 border where clustering of *fgf20a* neurons occurs, but expression is weaker in the other segments.

Loss of all segment boundaries leads to dispersal of *fgf20a* neurons

To further test the role of hindbrain boundary cells, we sought to disrupt their formation or maintenance independently of loss of Eph/ephrin signalling. Previous studies have shown that a deficiency

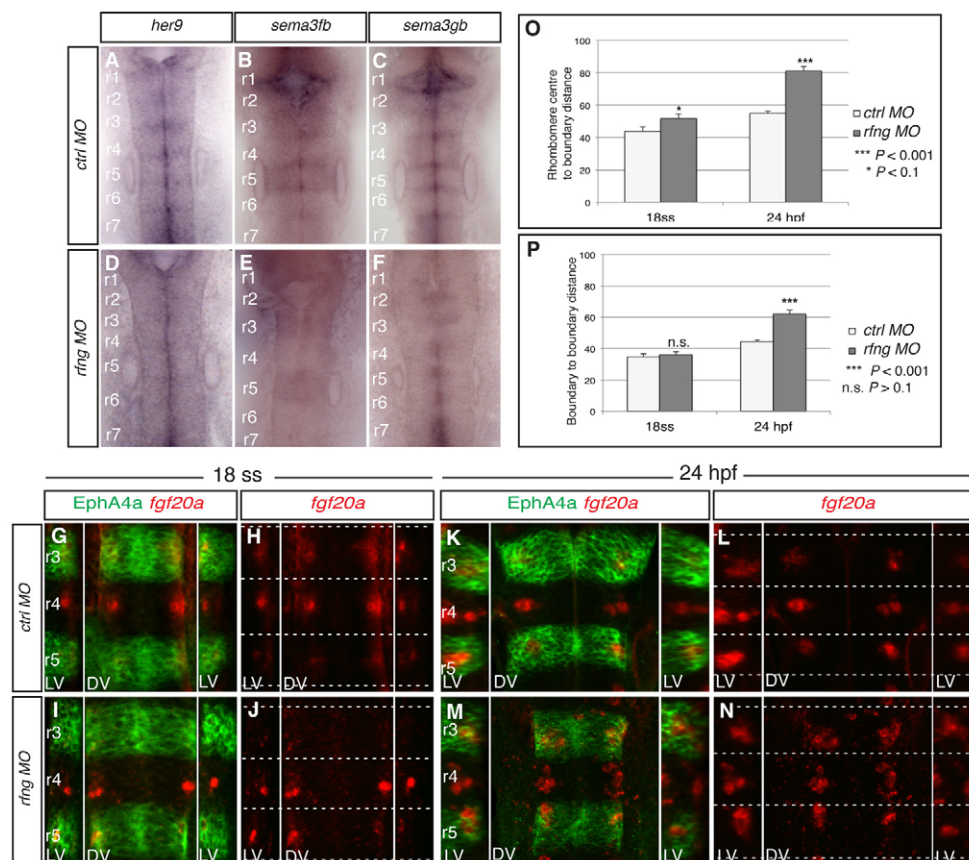


Fig. 2. Disruption of all rhombomere boundaries leads to spreading of *fgf20a* neurons. (A-F) Expression of *her9* (A,D), *sema3fb* (B,E) and *sema3gb* (C,F) in 24-hpf zebrafish embryos following injection of control MO (A-C) or *rfng* MO (D-F). *rfng* knockdown leads to loss of these markers at hindbrain boundaries (100%, *n*=14; 94%, *n*=16; 93%, *n*=15, respectively). (G-N) Expression of *fgf20a* mRNA (red) and EphA4a protein (green) at 18 ss (G-I) and 24 hpf (K-N) in control MO embryos (G,H,K,N) and *rfng* MO embryos (I,J,M,N). *rfng* knockdown has little effect on the organisation of *fgf20a* neurons at 18 ss (100%, *n*=8), but a strong effect at 24 hpf (90%, *n*=18). Dashed white lines indicate the position of segment borders. Left and right reconstructed lateral views (LV) and dorsal views (DV) are shown. (O) The average distance from *fgf20* neuronal cluster edge to boundary. y-axis represents the distance from the rhombomere centre (0) to the boundary (100 arbitrary units, A.U.). For control MO the average distance from centre to cluster edge at 18 ss is 43.8±2.8 A.U. (*n*=15) and at 24 hpf is 54.9±1.5 A.U. (*n*=48). For *rfng* MO the distance at 18 ss is 51.8±2.8 A.U. (*n*=30; *P*=0.09) and at 24 hpf is 81.2±2.6 A.U. (*n*=48; *P*=9.4×10⁻¹⁶). On average, clusters in *rfng* morphants are 18% closer to boundaries than in controls at 18 ss, which increases significantly to a difference of 48% by 24 hpf. (P) Average anteroposterior (AP) length of *fgf20* neuronal clusters. y-axis represents the distance from one rhombomere boundary (0) to another boundary (100 A.U.). For control MO at 18 ss the distance is 34.8±1.9 A.U. (*n*=15) and at 24 hpf is 44.6±0.9 A.U. (*n*=48). For *rfng* MO embryos the average length at 18 ss is 36.1±1.9 A.U. (*n*=30; *P*=0.67) and at 24 hpf is 62.1±2.4 A.U. (*n*=29; *P*=4.4×10⁻⁷). At 18 ss the difference is not significant, whereas at 24 hpf there is a significant 39% increase in length compared with control embryos. Values are average ± s.e.m. The orientation of embryos is as in Fig. 1.

of Notch activation leads to progressive depletion of hindbrain boundary cells, probably because Notch limits the differentiation of neural progenitors (Cheng et al., 2004; Qiu et al., 2009). *rfng* is a good candidate to be involved in boundary cell maintenance because its expression is restricted to rhombomere boundaries (Cheng et al., 2004; Qiu et al., 2004) and, by glycosylating Notch, Rfng promotes Notch activation in other contexts (Irvine and Wieschaus, 1994). We knocked down *rfng* and found that this leads to decreased boundary expression of *her9*, *sema3gb* and *sema3fb* (Fig. 2A-F), whereas borders of *epha4a* expression remain sharp (Fig. 2G,I). Unlike knockdown of *epha4a* or *efnb3b*, *rfng* knockdown disrupts boundary cell marker expression at all segment borders.

We found that *rfng* knockdown leads to alterations in the position of *fgf20a* neurons at 24 hpf (Fig. 2K-N) and in the pattern of *etv5b* expression, which becomes less localised to segment centres and/or decreases in level (supplementary material Fig. S2C). Whereas in control embryos the clusters of *fgf20a* neurons are at rhombomere centres (Fig. 2K,L), following *rfng* knockdown they are either closer to one of the boundaries or more spread out along the anteroposterior (AP) axis of the rhombomere (Fig. 2M,N). Quantitation of the number of *fgf20a* neurons revealed no significant change in *rfng* morphants (supplementary material Fig. S3A) and thus the altered organisation appears to be due to mispositioning rather than excess production. Unlike the situation in *efnb3b* morphants, we did not detect a systematic anterior or posterior movement of specific clusters. Since the organisation of *fgf20a* neurons in *rfng* morphants appeared normal at 18 ss (Fig. 2G-J), we conclude that boundaries are required to maintain rather than establish their positioning.

The altered distribution of *fgf20a* neurons varies between *rfng* morphants (supplementary material Fig. S4C) and even between the left and right sides of the same rhombomere (Fig. 2N). We quantitated and assessed the statistical significance of the changes (supplementary material Fig. 4A) by measuring the position of the anterior and posterior edges of the *fgf20a* neuron population in relation to the boundary-to-boundary length of each segment (which is not significantly altered in *rfng* morphants; supplementary material Fig. S3B). We used this to calculate the distance from the segment centre to the boundary occupied by *fgf20a* neurons. Whereas in control 24-hpf embryos *fgf20a* neurons occupy 55% of the centre-to-boundary distance, in *rfng* knockdown embryos they occupy 81% (Fig. 2O). We also measured the average length of the clusters along the AP axis and found that this increased from 45% in control embryos to 62% in *rfng* knockdown embryos (Fig. 2P). In contrast to this significant spreading at 24 hpf, there was little difference between control and *rfng* morphant embryos at 18 ss (Fig. 2O,P).

Semaphorins expressed at hindbrain boundaries maintain the positioning of *fgf20a* neurons

The migration of *fgf20a* neurons to the r5/r6 border following loss of *epha4a* or *efnb3b* function might be because boundary cells are depleted only at specific segment interfaces, as this phenotype is not seen following *rfng* knockdown, in which all boundaries are disrupted. If boundary cells are a source of a repellent cue, signalling from residual boundaries would act to relocate *fgf20a* neurons, whereas depletion of all boundaries would decrease chemorepulsion and lead to a spreading of these neurons. Specifically, this model can explain why in *epha4a* and *efnb3* knockdown embryos the *fgf20a* neurons in r5 and r6 move to the r5/r6 border, equidistant from the remaining boundary cells, although it does not account for the unaltered distribution of r4 neurons. This raises the question of

whether hindbrain boundary cells express any chemorepellents. Semaphorins are good candidates to fit this role of a repellent because, in addition to roles in axon guidance, they have been implicated in directing the migration of neuronal cell bodies (Tamagnone and Comoglio, 2004; Ayala et al., 2007; Marin et al., 2010). Two members of this ligand family, *sema3gb* and *sema3fb*, are specifically expressed in rhombomere boundaries (Fig. 2B,C) (Cooke et al., 2005). Furthermore, both *sema3gb* and *sema3fb* are downregulated, as with all boundary markers tested, following knockdown of *epha4a* (Cooke et al., 2005) or *rfng* (Fig. 2E,F). *sema3fb* is also segmentally expressed in r3 and r5, and this aspect of its expression is not altered following disruption of boundary cell formation (Fig. 2B,E).

We carried out knockdowns to determine whether *sema3gb* or *sema3fb* regulates the positioning of *fgf20a* neurons. We found that single knockdowns of *sema3gb* or *sema3fb* do not alter the organisation of *fgf20a* neurons, which, because these semaphorins act through the same receptor (Pellet-Many et al., 2008), could be due to overlapping functions. By contrast, knockdown of *sema3gb* plus *sema3fb* led to spreading or mispositioning of *fgf20a* neurons (Fig. 3A-D; supplementary material Fig. S4D) and decreased or mislocalised *etv5b* expression (supplementary material Fig. S2E,F),

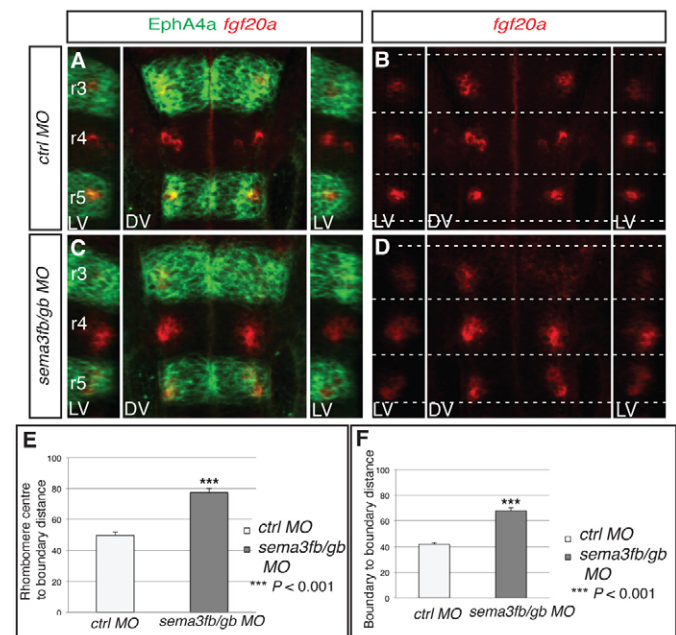


Fig. 3. Sema3fb and Sema3gb position *fgf20a* neurons.

(A-D) *fgf20a* expression (red) combined with EphA4 antibody staining (green) at 24 hpf in zebrafish embryos injected with control MO (A,B) or *sema3fb+sema3gb* MO (C,D). Dashed white lines indicate the position of boundary marker expression. Left and right reconstructed lateral views (LV) plus dorsal views (DV) are shown. Knockdown of *sema3fb+sema3gb* disrupts the organisation of *fgf20a* neurons (79%, $n=24$). (E,F) The average distance from *fgf20a* neuronal clusters to boundary (E) and average AP length of clusters (F); see Fig. 2 legend for methodology. In control MO embryos, the average distance to the boundary is 49.7 ± 2.1 A.U. ($n=24$); in *sema3fb+sema3gb* MO embryos this increases to 77.4 ± 2.8 A.U. ($n=24$; $P=2.2 \times 10^{-8}$), resulting in *sema3fb+sema3gb* clusters being on average half the distance from boundaries than control clusters. In control MO embryos the average cluster length is 41.8 ± 1.4 A.U. ($n=24$), which increases in *sema3fb+sema3gb* MO embryos to 68.0 ± 2.3 A.U. ($n=24$; $P=3.0 \times 10^{-9}$), a 62% increase in length. Values are average \pm s.e.m. The orientation of embryos is as in Fig. 1.

without any significant change in the number of these neurons (supplementary material Fig. S3A). Quantitation of the distribution of *fgf20a* neurons in *sema3fb+sema3gb* morphants reveals a significant increase in the distance that they occupy from segment centre to boundary and in the AP length of the cluster (Fig. 3E,F). The similar effect of knockdown of *sema3fb+sema3gb* and *rfng*, in which the clusters of *fgf20a* neurons are mislocalised or spread out towards boundaries, is consistent with these semaphorins acting as the proposed repellent cues. The remaining segmental expression of *sema3fb* in r3 and r5 might explain why the *fgf20a* neurons in r4 are not mislocalised in *epha4a* knockdown embryos despite the depletion of anterior boundary cells of this segment.

To further address the role of *sema3fb* and *sema3gb* in positioning *fgf20a* neurons, we analysed the expression and role of their receptors, *nrp2a* and *nrp2b*. We found that *nrp2a* expression at segment centres (Yu et al., 2004; Yu and Moens, 2005) is coincident with *fgf20a* expression, whereas *nrp2b* is not expressed (supplementary material Fig. S5). Following *nrp2a* knockdown (but not *nrp2b* knockdown), *fgf20a* neurons are more spread out and closer to the boundary (Fig. 4A-D, quantitation in 4E,F; supplementary material Fig. S4E), but do not significantly change in number (supplementary material Fig. S3A).

***sema3fb+sema3gb* or *nrp2a* knockdown alleviates *fgf20a* neuron migration induced by disrupting a subset of boundaries**

The finding that, following *epha4a* or *efnb3b* knockdown, *fgf20a* neurons in r5 and r6 become relocalised to the r5/r6 border might be explained by chemorepulsion from the boundary cells, which remain at the r4/r5 and r6/r7 interfaces. This model predicts that knockdown of *sema3fb+sema3gb* will rescue the mislocalisation of these cells following *efnb3b* knockdown. We carried out knockdown of *efnb3b* and visualised segment borders by detection of EphA4, and, as shown above, found that the *fgf20a*-expressing clusters in r5 and r6 are now located closer to the r5/r6 border at 24 hpf (Fig. 4I,J) than in controls (Fig. 4G,H). However, when *efnb3b* knockdown is combined with *sema3fb+sema3gb* knockdown, there is less movement of *fgf20a* neurons towards the r5/r6 border and the r5 and r6 clusters do not collide (Fig. 4K,L, quantitated in 4O). Instead, the *fgf20a* neurons are more spread out both anteriorly and posteriorly, a characteristic of knockdown of *sema3fb+sema3gb*. Similarly, *nrp2a* knockdown decreased the movement of *fgf20a* neurons towards the r5/r6 border in *efnb3b* morphants (Fig. 4M-O). These results confirm that *Sema3fb* and *Sema3gb* act through their common receptor *Nrp2a* as repellent cues that position *fgf20a* neuronal clusters at segment centres.

Dispersal of *fgf20a* neurons leads to increased neurogenesis

Previous work showing that *fgf20a* suppresses neurogenesis at segment centres (Gonzalez-Quevedo et al., 2010) implies that the appropriate positioning of *fgf20a* neurons is required to achieve the correct patterning of neurogenesis. Indeed, we find that knockdown of *rfng*, *sema3fb+sema3gb*, or *nrp2a* each leads to disorganisation of the non-neurogenic zone, as marked by a lack of *neurogl* expression (Fig. 5A-D). To examine whether the dispersal of *fgf20a* neurons following loss of hindbrain boundary cells affects the overall amount of neurogenesis, we detected HuC/D (Elavl3/4) protein, which marks all differentiating neurons in the mantle zone, and quantitated the number of expressing cells in confocal stacks throughout the dorsoventral axis. We found that knockdown of *rfng*, *sema3fb+sema3gb*, or *nrp2a* led to a 30-60% increase in the number

of neurons (Fig. 5E-H, quantitated in 5M). The pattern of HuC/D staining in these morphants retains a segmented distribution, with a gap around hindbrain boundaries; this is likely to reflect persistence of the curtains of radial glial fibres that flank boundaries and physically exclude neurons (Trevarrow et al., 1990).

A potential explanation for the increased neurogenesis following the dispersal of *fgf20a* neurons is suggested by the observation that, in *epha4a* and *efnb3b* knockdown embryos, expression of the FGF target gene *etv5b* occurs at high levels in r4 and at the r5/r6 border, but at lower levels in all other hindbrain segments (Fig. 1P,Q). This pattern of *etv5b* expression correlates with the clustering of *fgf20a* neurons in r4 and the r5/r6 border, and suggests that the dispersal of these neurons in the other segments leads to a lower level of FGF pathway activation. We quantified HuC/D-expressing neurons within hindbrain segments following *efnb3b* knockdown and found a significant increase in r3 (13%) and decrease in r5 (13%), whereas r4 was unaffected (Fig. 5I-L, quantitated in 5N). The amount of neurogenesis thus correlates with the organisation of *fgf20a* neurons, which in *efnb3b* morphants are dispersed in r3, clustered normally in r4, and form a fused cluster of r5 and r6 neurons at the r5/r6 border.

DISCUSSION

Previous studies have shown that neurogenesis is spatially organised within hindbrain segments in zebrafish through the FGF-mediated inhibition of neurogenesis (Gonzalez-Quevedo et al., 2010). This inhibition is due to signalling from the mantle zone by *fgf20a* neurons located at each segment centre that underlies the formation of a non-neurogenic zone in the adjacent progenitor cells. These findings suggested that the stereotyped location of *fgf20a* neurons at segment centres might be important for the patterning of neurogenesis, and raise the question of how this positioning is achieved. Here, we have uncovered a mechanism in which signalling from hindbrain boundaries maintains the tight clustering and positioning of *fgf20a* neurons at segment centres. We show that selective loss of specific boundaries due to *epha4a* or *efnb3b* knockdown leads to an altered organisation of *fgf20a* neurons, including an abnormal migration of these neurons in r5 and r6 away from the remaining boundaries and towards the deficient r5/r6 border. This mispositioning of *fgf20a* neurons is matched by an altered pattern of FGF-dependent *etv5b* gene expression in the adjacent progenitor cells. Furthermore, loss of hindbrain boundary cells at all segment borders following *rfng* knockdown leads to a spreading and mispositioning of *fgf20a* neurons. The finding that the organisation of *fgf20a* neurons in *efnb3b* and *rfng* morphants appears normal at early stages argues that boundaries serve to maintain rather than establish the position of these neuronal clusters at segment centres. These observations also argue against a model in which boundaries regulate the organisation of *fgf20a* neurons by establishing a prepattern of their precursors in the neural epithelium.

The results of loss-of-function experiments implicate *Sema3fb* and *Sema3gb*, which are expressed in hindbrain boundaries, in positioning *fgf20a* neurons by acting through their common receptor *Nrp2a*. Consistent with this, we find that *sema3fb+sema3gb* knockdown alleviates the abnormal migration of *fgf20a* neurons towards the depleted r5/r6 boundary in *efnb3b* morphants. These findings are consistent with a model in which hindbrain boundary cells are a source of semaphorin signals that chemorepel *fgf20a* neurons towards segment centres where they are furthest from the flanking borders, leading to inhibition of neurogenesis in the adjacent progenitors (Fig. 6A,B). The tight

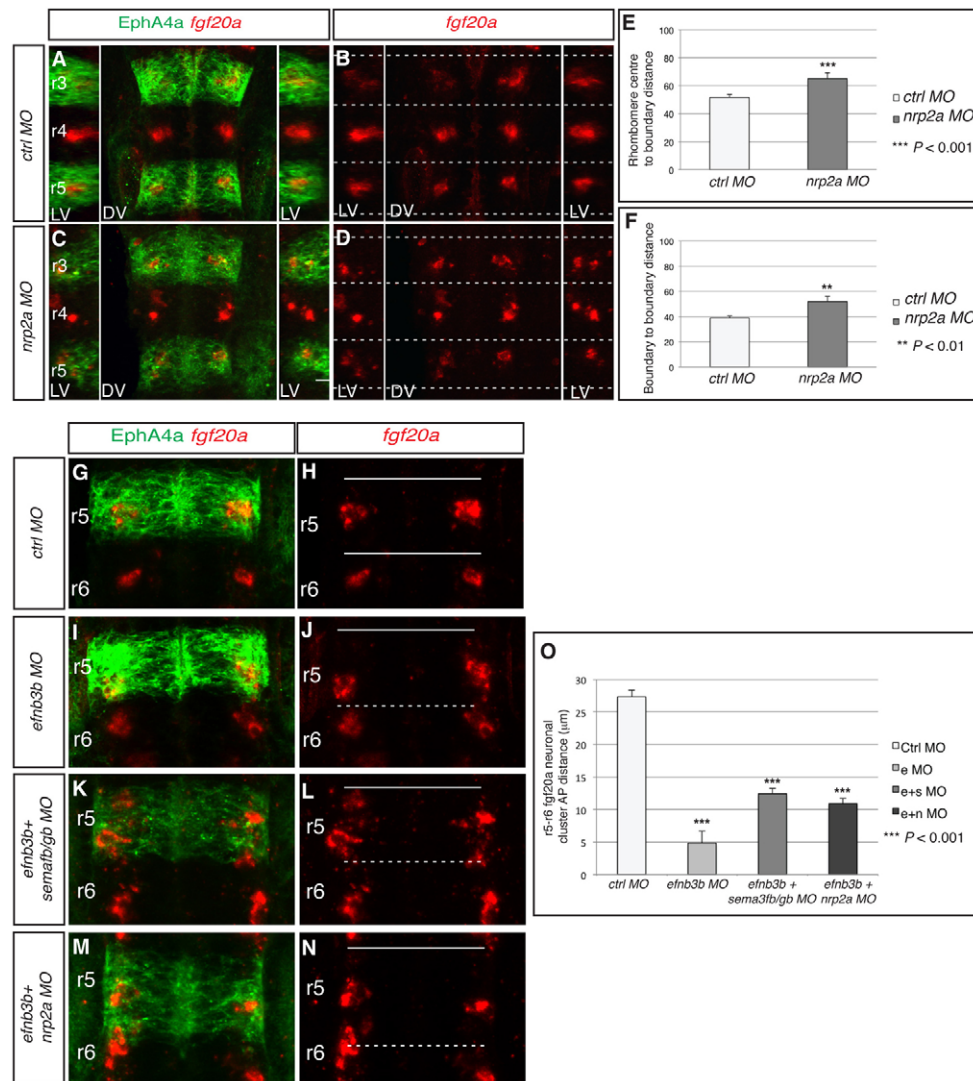


Fig. 4. Nrp2a is required to position *fgf20a* neuronal clusters. (A–D) *fgf20a* expression (red) combined with EphA4 antibody staining (green) at 24 hpf in zebrafish embryos injected with (A,B) control MO or (C,D) *nrp2a* MO. Dashed white lines indicate the position of segment borders. Left and right reconstructed lateral views (LV) plus dorsal views (DV). *nrp2a* knockdown disrupts the organisation of *fgf20a* neurons (70%, $n=17$). (E,F) The average distance from *fgf20a* neuronal clusters to boundary (E) and average AP length of clusters (F). In control MO embryos, the average distance to the boundary is 51.6 ± 2.2 A.U. ($n=24$), which in *nrp2a* MO embryos increases to 65.1 ± 4.1 A.U. ($n=24$; $P=5 \times 10^{-4}$), i.e. 22% closer to boundaries. In control MO embryos the average cluster length is 38.8 ± 1.6 A.U. and this increases in *nrp2a* morphants to 52.0 ± 4.0 A.U. ($n=24$; $P=4 \times 10^{-3}$), a 34% increase in length. (G–N) *fgf20a* mRNA expression (red) and EphA4 antibody staining (green) of embryos injected with control MO (G,H), *efnb3b* MO (I,J), *efnb3b+sema3fb+sema3gb* MOs (K,L) or *efnb3b+nrp2a* MOs (M,N). Dashed white lines indicate the position of depleted boundary cells; continuous white lines indicate the position of the remaining boundaries. The migration of *fgf20a* neurons towards the r5/r6 interface following *efnb3b* knockdown was partly blocked when combined with *sema3fb+sema3gb* knockdown (76%, $n=17$) or *nrp2a* knockdown (70%, $n=23$). (O) Quantitation of the distance between r5 and r6 *fgf20a* neuronal clusters in the different knockdown experiments. There is a significant decrease in the distance between both clusters in *efnb3b* morphants (e MO), as compared with control MO, that is significantly rescued by co-injection of *sema3fb+sema3gb* MOs (e+s MO) or *nrp2a* MO (e+n MO): control MO $27.3 \pm 1.0 \mu\text{m}$ ($n=10$) versus *efnb3b* MO $4.9 \pm 1.6 \mu\text{m}$ ($n=10$), $P=3.1 \times 10^{-9}$; e+s MO $12.4 \pm 0.8 \mu\text{m}$ ($n=10$), $P=9.5 \times 10^{-10}$; e+n MO $10.9 \pm 0.8 \mu\text{m}$ ($n=10$), $P=2.0 \times 10^{-10}$. Values are average \pm s.e.m.

clustering of *fgf20a* neurons has an important role in the patterning of neurogenesis: when it is disrupted following knockdown of *rtnf*, *sema3fb+sema3gb* or *nrp2a* the organisation of the neurogenic and non-neurogenic zones is altered (Fig. 6C,D).

Our findings raise the question of how the cluster of *fgf20a* neurons spreads out and becomes mislocalised in a variable pattern following the disruption of hindbrain boundaries or of *sema3fb/sema3gb* function. This might be associated with the major expansion of the mantle region as differentiating neurons migrate in

from the neural epithelium, as well as with the overall growth of neural tissue. This growth has the potential to drive cell intercalation that separates neighbours, such that repeated rounds of intercalation lead to a variable spreading of cells that are initially clustered.

Roles of boundaries in patterning

One of the important roles of boundary formation during development is to generate specialised boundary cells that are a source of inductive signals that regulate cell differentiation in a

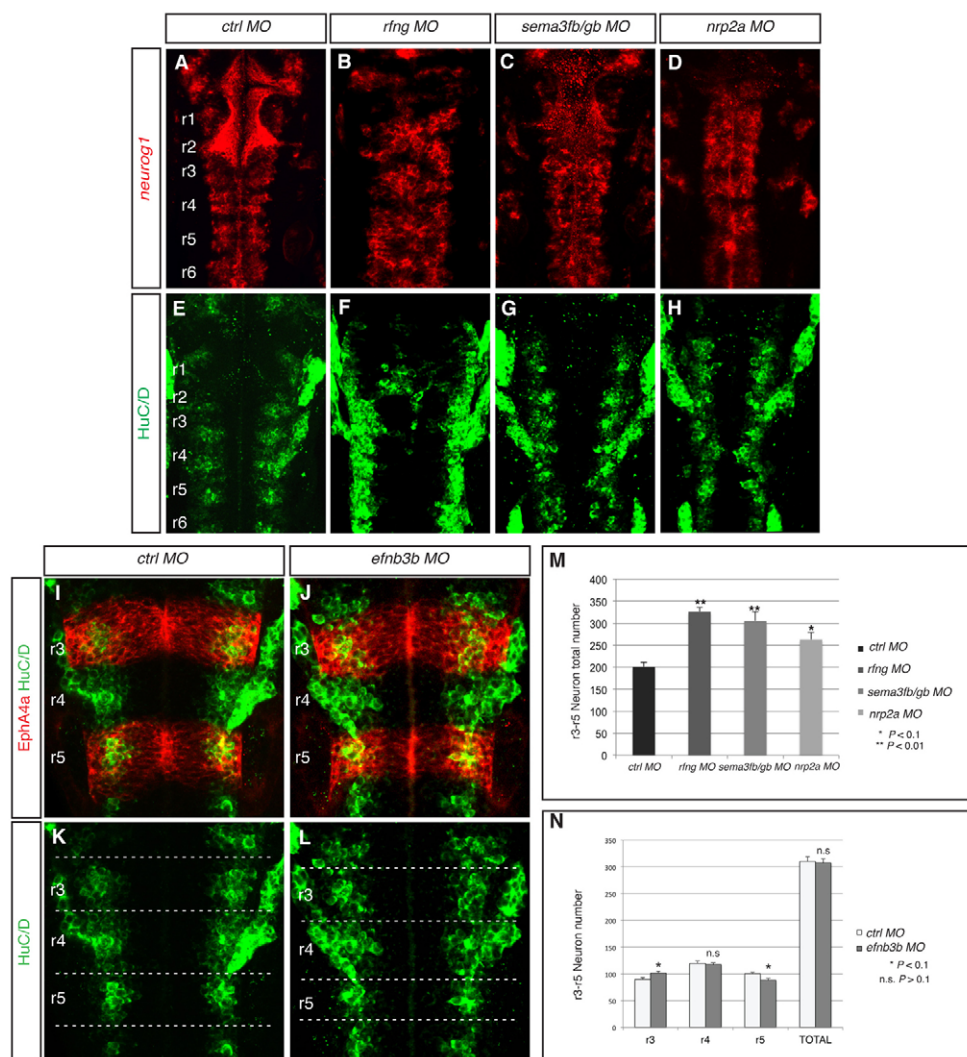


Fig. 5. Mispositioning of *fgf20a* neurons affects the patterning and amount of neurogenesis. (A-H,M) Expression at 30 hpf of *neurog1*, which marks differentiating neurons in the neural epithelium (red), and HuC/D, which marks neurons in the mantle zone (green), in embryos injected with control MO (A,E), *rfng* MO (B,F), *sema3fb+sema3gb* MOs (C,G) or *nrp2a* MO (D,H). Knockdown of *rfng* (85%, $n=14$), *sema3fb+sema3gb* (65%, $n=23$) or *nrp2a* (59%, $n=17$) leads to disorganisation of the neurogenic zones. (M) Quantitation of the number of HuC/D-expressing cells in r3 to r5 in control MO embryos (201 ± 9.7 neurons, $n=4$) reveals that these increase following knockdown of *rfng* (326 ± 16.2 neurons, $n=4$; $P=0.008$), *sema3fb+sema3gb* (306 ± 9.4 neurons, $n=4$; $P=0.001$) or *nrp2a* (263 ± 9.4 neurons, $n=4$; $P=0.06$). (I-L,N) HuC/D-expressing neurons (green) combined with EphA4 antibody staining (red) at 30 hpf in control (I,K) and *efnb3b* (J,L) knockdown embryos. (N) Quantitation of neurons in both conditions in r3, r4 and r5 separately plus total number. There is a significant increase in the number of neurons in r3 (control MO 90 ± 2.7 , $n=5$, versus *efnb3b* MO 101 ± 3.1 ; $P=0.09$), a decrease in r5 (control MO 100 ± 2.7 , $n=5$, versus *efnb3b* MO 88 ± 3.5 ; $P=0.06$), but no significant change in r4 (control MO 120 ± 4.6 , $n=5$, versus *efnb3b* MO 118 ± 3.3 ; $P=0.74$). The total number of neurons is not significantly different (control MO 310 ± 9.1 , $n=5$, versus *efnb3b* MO 307 ± 7.2 ; $P=0.85$). The orientation of embryos is as in Fig. 1.

concentration-dependent manner (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; Kiecker and Lumsden, 2005). Our findings reveal a distinctive role of hindbrain boundaries in which, by acting as a source of chemorepellent, they serve to position another signalling source – *fgf20a* neurons – that patterns cell differentiation. It remains possible that other signals emanating from hindbrain boundaries have an inductive role in which they promote the differentiation of adjacent progenitors, such that the neurogenic zone is positioned by antagonistic inductive and inhibitory signals. However, this seems unlikely because neurogenesis does not decrease – on the contrary, it increases – following depletion of boundary cells. Thus, other boundary signals, such as Wnt family members (Riley et al.,

2004; Amoyel et al., 2005), are not essential for neuronal differentiation and might regulate other aspects of hindbrain development.

Neuronal organisation and neurogenesis

The precise positioning of neuronal cell types and their organisation into clusters are ubiquitous aspects of nervous system development. This positioning requires the radial migration of neuronal cell bodies from the neural epithelium, and in some cases tangential migration over long distances, guided by contact-dependent or diffusible cues that include semaphorins (Ayala et al., 2007; Marin et al., 2010). The significance of such migration to position neuronal cell bodies at a particular location is unclear, but

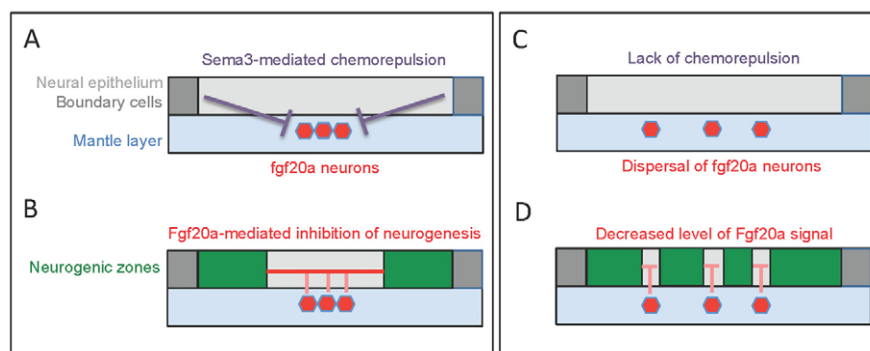


Fig. 6. Model of the role of boundaries in neuronal positioning. A single zebrafish hindbrain segment is represented with the neural epithelium in light grey, boundary cells in dark grey, and the mantle layer in blue. (A,B) Normal embryos, in which Sema3fb/Sema3gb-mediated chemorepulsion from boundary cells positions *fgf20a* neurons (red) in the segment centre. The cluster of *fgf20a* neurons creates a focussed source of FGF signals (red lines) that inhibit neurogenesis in the adjacent neural epithelium, restricting the neurogenic zones (green) to being adjacent to boundaries. (C,D) Embryos in which boundary cells have been depleted or Sema3fb/Sema3gb signalling disrupted. The consequent dispersal of *fgf20a* neurons leads to disorganisation of the non-neurogenic zones and diminishes the level of FGF signalling.

is thought to facilitate the formation of functional neuronal circuits (Chen et al., 2006). In the case of reticulospinal neurons, which are located at segment centres in the hindbrain (Hanneman et al., 1988), these are components of an early neuronal circuit that underlies an escape response (Liu and Fetcho, 1999). Our findings suggest another type of role for neuronal clustering.

We find that depletion of hindbrain boundary cells leads to a significant increase in the overall amount of neurogenesis. One potential explanation is that signals from boundary cells inhibit neurogenesis, albeit that this requires other factors to account for the normal formation of neurogenic zones adjacent to boundaries. However, increased neurogenesis also occurs following disruption of Sema3fb/Sema3gb signalling, in which hindbrain boundary cells remain present. These findings argue for a model in which the clustering of *fgf20a* neurons is required for the correct patterning and amount of neurogenesis. We find that the spreading of *fgf20a* neurons along the AP axis that occurs following the disruption of boundaries or of Sema3fb/Sema3gb signalling does not lead to a wider non-neurogenic zone, but rather correlates with an increase in the overall amount of neurogenesis. A potential explanation is suggested by the observation that, following *epha4* or *efnb3b* knockdown, expression of the FGF-response gene *etv5b* remains at a high level in r4 and at the r5/r6 border where clustering of *fgf20a* neurons still occurs, but is at lower levels in all of the other segments. This suggests that clustering of Fgf20a signalling cells is required to achieve a high level of FGF signal and target gene activation. We therefore propose that neuronal differentiation is inhibited above a threshold level of FGF pathway activation in progenitor cells, and that this requires the tight clustering of *fgf20a* neurons to provide a focussed source of FGF signals. This is reminiscent of the situation for signalling centres that are induced at boundaries, which require that the border is sharp and straight in order to achieve the appropriate distribution of the graded signal (Dahmann and Basler, 1999). Our findings thus provide a further perspective on the mechanistic importance of establishing a precise organisation of signalling cells and how this can be achieved.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.080135/-DC1>

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