Lunatic fringe promotes the lateral inhibition of neurogenesis

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Previous studies have identified roles of the modulation of Notch activation by Fringe homologues in boundary formation and in regulating the differentiation of vertebrate thymocytes and Drosophila glial cells. We have investigated the role of Lunatic fringe (Lfng) expression during neurogenesis in the vertebrate neural tube. We find that in the zebrafish hindbrain, Lfng is expressed by progenitors in neurogenic regions and downregulated in cells that have initiated neuronal differentiation. Lfng is required cell autonomously in neural epithelial cells to limit the amount of neurogenesis and to maintain progenitors. By contrast, Lfng is not required for the role of Notch in interneuronal fate choice, which we show is mediated by Notch1a. The expression of Lfng does not require Notch activity, but rather is regulated downstream of proneural genes that are widely expressed by neural progenitors. These findings suggest that Lfng acts in a feedback loop downstream of proneural genes, which, by promoting Notch activation, maintains the sensitivity of progenitors to lateral inhibition and thus limits further proneural upregulation.

KEY WORDS: Lateral inhibition, Neurogenesis, Neural progenitors, Notch, Fringe, Zebrafish

INTRODUCTION

Intercellular signalling mediated by the Notch receptor has diverse roles in the regulation of cell differentiation, proliferation and migration during development (Louvi and Artavanis-Tsakonas, 2006). The response of cells to Notch activation is context dependent and can change within a tissue at different stages of development. This is illustrated by roles of Notch in cell differentiation in the vertebrate central nervous system, in which it mediates the lateral inhibition of neurogenesis (Lewis, 1998), promotes the formation of radial glial cells (Gaiano et al., 2000), which can act as neural progenitors (Malatesta et al., 2000; Malatesta et al., 2003), and regulates the choice to differentiate into specific neuronal cell types (Peng et al., 2007). A general feature of these functions is that Notch signalling diversifies adjacent cells by lateral inhibition or induction, in which cells expressing Notch ligands change the differentiation of their neighbours.

During the lateral inhibition of neurogenesis in the vertebrate nervous system, proneural transcription factors that drive the initial steps of neuronal differentiation upregulate expression of the Notch ligands, Delta or Serrate/Jagged. These ligands activate Notch in adjacent cells by promoting a proteolytic cleavage that releases the intracellular domain of Notch (Mumm et al., 2000), which, upon binding to the transcription factor CSL, switches it from a repressor to an activator (Fryer et al., 2002). The activated CSL complex upregulates expression of members of the Hes/Her transcriptional repressor family that inhibit neurogenesis (Kageyama et al., 2007). Consequently, a pool of undifferentiated cells is maintained adjacent to differentiating neurons, within which neurogenesis can be initiated once lateral inhibition is relieved as the forming neuron migrates away from the neural epithelium.

In addition to roles in controlling cell differentiation, in some tissues Notch activation contributes to the inhibition of cell intermingling across boundaries (Dominguez and de Celis, 1998; Michelli and Blair, 1999; Papayannopoulos et al., 1998). An example is the dorsoventral boundary in the Drosophila wing imaginal disc, in which the role of Notch depends upon the glycosyltransferase Fringe (Kim et al., 1995; Rauskolb et al., 1999). Fringe glycosylates specific sites of the Notch extracellular domain during its intracellular processing, and this glycosylation alters the affinity of Notch binding to its ligands: Delta binds more strongly to Fringe-modified Notch, whereas the binding of Serrate is decreased (Moloney et al., 2000; Panin et al., 1997). Consequently, the dorsal expression of Fringe and Serrate and the ventral expression of Delta leads to a stripe of Notch activation at the dorsoventral interface, which is required to form the compartment boundary (de Celis et al., 1996). Studies of vertebrate homologues of Fringe have suggested analogous roles in boundary formation for lunatic fringe (Lfng) in the chick forebrain (Zeltser et al., 2001), and for radical fringe in the chick limb (Lauffer et al., 1997; Rodriguez-Esteban et al., 1997). Similarly, the expression of Radical fringe by zebrafish hindbrain boundary cells may modulate Notch activation that then regulates cell segregation (Cheng et al., 2004).

Some contexts have been found in which Fringe homologues are involved in the regulation of cell differentiation. During lymphopoiesis, Lfng expression in thymocyte progenitors promotes their differentiation by increasing the activation of Notch by limiting amounts of Delta expressed in thymic epithelial cells (Visan et al., 2006). Similarly, in the Drosophila CNS, Fringe is upregulated in specific glial cells and promotes Notch activation required for subtype-specific gene expression (Thomas and van Meyel, 2007). It is therefore intriguing that specific Fringe homologues are expressed in the vertebrate CNS; for example, Lfng expression occurs in dorsoventral stripes in the chick and mouse neural tube (Johnston et al., 1997; Lauffer et al., 1997) that could correlate with neurogenesis, and Fringe homologues are expressed in progenitors and...
differentiating cells in the cerebral cortex in mouse (Ishii et al., 2000). Furthermore, overexpression of Lfng in the chick neural tube was found to increase the number of neurons (de Bellard et al., 2007). These findings raise the possibility that modulation of Notch activity by Fringe homologues regulates neurogenesis in vertebrates.

We set out to investigate the role of Lfng in the zebrafish nervous system, in which gene expression studies have suggested potential roles in boundary formation and/or neurogenesis. At early stages, lfg expression occurs at high levels in alternating segments in the hindbrain (Leve et al., 2001; Prince et al., 2001; Qiu et al., 2004), which by analogy with roles in other tissues could underlie boundary formation. In addition, lfg is expressed in dorsallyventrally restricted domains in the neural tube (Prince et al., 2001) that could be associated with zones of neurogenesis. We show that Lfng limits neuronal differentiation and is required to maintain progenitor cells. Lfng acts cell autonomously in progenitors to inhibit their differentiation but, surprisingly, is upregulated downstream of proneural genes. We propose that Lfng acts in a feedback loop that maintains the competence of progenitor cells to receive lateral inhibition from differentiating neurons.

MATERIALS AND METHODS

Zebrafish lines
Wild-type, mibta52 (Jiang et al., 1996; Schier et al., 1996), notch1a<sup>−/−</sup> (Gray et al., 2001; Holley et al., 2002; van Eeden et al., 1996) and Tg(r3/r5-Gal4::UAS-RFP) embryos were produced and staged according to hours post fertilisation (hpf) and morphological criteria (Kimmel et al., 1995).

Microinjection
Liberomes (1- to 4-cell) were microinjected with 0.45-1.8 pmol morpholino oligonucleotide (MO; Gene Tools). The lfg splice-blocking MO (ACCGTGTATACCTGTGGCATCTTTC) was used as control. To test the effect on splicing, RT-PCR was performed with ~20 24 hpf embryos using the primers P1, 5′-GGTTTCTGTGTTTCTCTGGCAG-3′; P2, 5′-CTCAGCGCTTGTGAAGATGTA-3′; and P3, 5′-CTTTATATGGGT-TTTGTGGTACAGC-3′. For proneural knockdown, ascl1a, ascl1b and ngnl MOs (Amoyel et al., 2005) were used. When used, 0.45 pmol of p35 MO (Robu et al., 2007) was coinjected. Capped RNA encoding ngnl-myc, dominant-negative CSL [MO (Robu et al., 2007)] or dominant-negative DBM [Tg(e1b-Gal4::UAS-RFP)] embryos were produced and staged according to hours post fertilisation (hpf) and morphological criteria (Kimmel et al., 1995).

RESULTS

The expression of lfg is associated with segmentation and neurogenesis
Previous studies have reported that at 12.5-14 hpf, lfg is expressed at higher levels in rhombomeres (r) 2 and 4 in the hindbrain, and by 20 hpf has been upregulated in other segments (Leve et al., 2001; Prince et al., 2001; Qiu et al., 2004). In a more extensive analysis, we observed that lfg expression is at higher levels in r2 and r4 at 14-18 hpf (Fig. 1A,B), by 24 hpf occurs in a punctate pattern (Fig. 1C), and then becomes restricted to zones adjacent to hindbrain boundaries (Fig. 1D,E). This later phase of expression is reminiscent of the pattern of neurogenesis in the zebrafish hindbrain (Amoyel et al., 2005). We therefore investigated whether Lfng has roles in hindbrain boundary formation and/or in neurogenesis.

Knockdown of lfg does not affect segmentation or boundary formation
We carried out loss-of-function experiments by using a morpholino oligonucleotide (MO) complementary to the splice donor site of intron 1 of lfg (lfng<sup>E1I1</sup> MO) (Fig. S1A in the supplementary material); this is predicted to block splicing of intron 1, and then become restricted to zones adjacent to hindbrain boundaries (Fig. 1D,E). This later phase of expression is reminiscent of the pattern of neurogenesis in the zebrafish hindbrain (Amoyel et al., 2005). We therefore investigated whether Lfng has roles in hindbrain boundary formation and/or in neurogenesis.
of sharp borders of segmental markers following *lfng* knockdown (see Fig. S2 in the supplementary material). Lfng therefore does not appear to be required for these processes.

**Lfng limits the amount of neurogenesis**

To address whether Lfng has a role in the regulation of neurogenesis, we analysed the effect of *lfng* knockdown on the expression of proneural and Delta genes that are upregulated at the onset of neuronal differentiation (Allende and Weinberg, 1994; Haddon et al., 1998; Korzh et al., 1998), on neurod4, which is upregulated downstream of proneural genes (Park et al., 2003; Wang et al., 2003), and on HuC/D, which marks differentiating neurons in the mantle zone (Park et al., 2000). We found that *lfng* knockdown leads to increased expression of proneural and Delta genes, including ngn1 (neurog1 – ZFIN), ascl1a, ascl1b, deltaA and deltaB, and of neurod4 at all stages analysed, for example at 18 and 28 hpf (see Fig. S3 in the supplementary material) and 36 hpf (Fig. 2A-L). This increase appeared to be due in part to a higher level of gene expression per cell, as more intense signals are observed at single cell resolution following *lfng* knockdown (see, for example, Fig. 2A-H11032-LH11032). In addition, there was an increased number of cells expressing high levels of proneural and Delta genes, seen for example at 36 hpf, when neurogenesis is confined to narrow neurogenic zones: in control embryos there is a mixture of low- and high-expressing cells within the ventral and mantle zones. Scale bars: 100 μm in A for A-E; 10 μm in F for F-G; 50 μm in H for H-I.

![Fig. 1. Expression pattern of *lfng* in the zebrafish hindbrain.](image)

**Fig. 2. Knockdown of *lfng* increases the initiation of neuronal differentiation.** Dorsal views of control MO (A-F) and *lfng* MO (G-L) injected embryos at 36 hpf showing ngn1 (A,A’H11032,G,G’H11032), ascl1a (B,B’H11032,H11032), ascl1b (C,C’H11032,J,J’H11032), deltaA (D,D’J11032), deltaB (E,E’K,K’H11032), and neurod4 (F,F’L11032) expression in the hindbrain. A’-L’ are higher magnifications of the indicated areas in A-L. Scale bar: 100 μm.
these zones, whereas following lfn MO knockdown there are more cells with high level expression (Fig. 2B'-D', H'-J'). Consistent with an increased number of cells initiating neurogenesis, we observed more differentiating neurons in the mantle zone marked by HuC/D (Fig. 3A-I). In order to quantify the effect on neuronal differentiation, we compared the number of HuC/D-positive cells in control and lfn MO embryos. We found that lfn knockdown leads to a 1.7-fold increase in the number of differentiating neurons, both at 18 and 30 hpf (Fig. 3K and data not shown).

These findings raised the question of whether Lfn limits the production of all types of neurons in the hindbrain, or acts in a specific subset. To examine this, we analysed markers of different neuronal types, including interneurons, and reticulospinal, branchiomotor, somatic motor and commissural neurons. We found that there was an increase in the number of all neuronal types examined, apart from reticulospinal neurons (see Fig. S4 in the supplementary material). These findings reveal that Lfn has a widespread role in limiting neurogenesis, with the exception of reticulospinal neurons.

A potential difficulty with MOs is that they can have off-target effects leading to p53-mediated cell death, although this can be suppressed by coinjection with p53 MO (Robu et al., 2007). Since such apoptosis leads to the loss of cells, it seemed unlikely that this underlies the observed increase in neurogenesis. Nevertheless, we tested the effect of coinjecting p53 MO and found that this does not alter the increase in neurogenesis following injection of lfn MO (data not shown). In addition, we assessed the specificity of the phenotypic effect of lfn MO in a rescue experiment in which Lfn is transgenically overexpressed in r3/r5 using a Gal4 enhancer-trap line (Fig. 3LJ). We found that transgenic overexpression of Lfn decreased the amount of neurogenesis in control embryos and partly suppressed the lfn MO-induced increase in neurogenesis (Fig. 3K); both of these effects specifically occurred in r3/r5, where Lfn is ectopically expressed, and not in r4.

**Lfn is required to maintain progenitors**

A potential explanation for our findings is that lfn knockdown leads to a deficiency in the lateral inhibition of neurogenesis. We therefore analysed expression of Her3/Her family genes that are targets of the Notch pathway during lateral inhibition. We observed no change in the expression of her4 (her4.1 – ZFIN) at 28 hpf (see Fig. S3 in the supplementary material), whereas by 40 hpf the expression of her12 and her12 was decreased following lfn knockdown (Fig. 4A,B,E,F). This appeared to be due to a decrease in the levels of expression, as well as in the number of cells expressing the Notch effectors (Fig. 4A',B',E',F'). However, her4 expression may not provide a sensitive read-out of Notch modulation since, as occurs for the homologous genes in Drosophila (Castro et al., 2005), its expression may also be upregulated by proneural genes (Yeo et al., 2007); the upregulation of proneural genes following lfn knockdown could thus mask any decrease in Notch activation. A potential explanation for the late decrease in her4 and her12 gene expression is that the reduced lateral inhibition of neurogenesis led to a depletion of progenitors, so we analysed the expression of sox3 and sox19a, which mark neural progenitor cells. We found that, although no change was detected at 28 hpf (see Fig. S3 in the supplementary material), by 40 hpf there was lower expression of these markers, which is suggestive of fewer neural progenitors in the neural tube (Fig. 4C',D',G',H'). Taken together, these results suggest that knockdown of lfn eventually leads to a depletion of progenitor cells, consistent with a role in Notch-mediated lateral inhibition of neurogenesis.

**Lfn is not required for neuronal subtype specification**

Recent work has shown that, in addition to being required in progenitors during the lateral inhibition of differentiation, Notch1 activation regulates the choice to form V2b rather than V2a interneurons in the spinal cord (Batista et al., 2008; Del Barrio et al., 2007; Peng et al., 2007). This raises the possibility that, in addition to a role in the inhibition of neurogenesis, Lfn enables the activation of Notch required for this fate choice. We therefore analysed whether Notch1 is required for interneuron fate choice in the zebrafish hindbrain. We found that, whereas notch1b and notch3 are widely expressed in the nervous system, notch1a expression occurs initially at higher levels in r2 and r4 and subsequently in neurogenic zones (see Fig. S5 in the supplementary material).
Furthermore, unlike other Notch receptors (data not shown), notch1a is coexpressed with Delta genes (see Fig. S6 in the supplementary material) and upregulated in neurogenic mib mutants (Fig. 5A-D). We therefore analysed whether interneuron subtype choice regulated by Notch signalling is altered in the notch1ap37 mutant. Indeed, we found that there is a loss of V2b and an increase in V2a interneurons in this mutant (Fig. 5E,F,I,J), as occurs following global Notch inhibition (Batista et al., 2008; Peng et al., 2007).

If Lfng is required for Notch1a function in differentiating neurons, knockdown of lfng would have a similar effect on neuronal subtype specification as occurs in the notch1ap37 mutant. However, we found that lfng knockdown does not lead to a switch in the fate of interneuron subtypes, but rather increases the numbers of both interneuron populations (Fig. 5G,H,K,L). We therefore conclude that Lfng is required to promote the lateral inhibition of neurogenesis but not for interneuron subtype specification.

The expression of lfng is regulated by proneural genes

In order to understand how Lfng contributes to the inhibition of neurogenesis, it is essential to determine in which cells lfng is expressed and how its expression is regulated. For example, lfng could be upregulated by Notch activation in progenitors and/or by proneural genes that are widely expressed at low levels and upregulated in differentiating neurons. We therefore compared neuronal marker and lfng expression at single cell resolution. deltaA is widely expressed at low levels in progenitors, and upregulated in cells selected to differentiate during lateral inhibition, whereas deltaB is only expressed in cells that have initiated differentiation (Haddon et al., 1998). In confocal sections in a superficial plane of the neural epithelium we found that lfng overlaps with deltaA expression (Fig. 1F,G). To visualise how lfng expression relates to the transition from progenitors to differentiating neurons, we analysed transverse hindbrain sections and found that lfng expression is confined to the ventricular zone, where it overlaps with the low- and high-level expression of deltaA (Fig. 1H). By contrast, lfng expression occurs complementary to the expression of deltaB in differentiating neurons migrating to the mantle zone (Fig. 1I). We conclude that lfng is coexpressed with Delta genes in neural progenitors and is downregulated in cells that have embarked upon neurogenesis.

To test whether lfng is regulated by proneural genes and/or by Notch activation, we first carried out knockdown and gain-of-function experiments with proneural genes. Knockdown of ngn1 or ascl1b alone had only a moderate effect on lfng expression, as was also seen for deltaA expression (see Fig. S6 in the supplementary material). By contrast, triple knockdown of ngn1, ascl1b and ascl1a led to a major decrease in lfng expression (Fig. 6A,E and see Fig. S6 in the supplementary material). In gain-of-function experiments, we found that misexpression of ngn1 (Fig. 6G,I,K,M), but not of ascl1b (data not shown), led to upregulation of lfng expression. Taken together, these observations suggest that lfng is a downstream target of multiple proneural genes that can compensate for each other following knockdown, but there are differences between proneural genes in their ability to upregulate lfng.
An alternative explanation for the effects of proneural knockdown or misexpression on *lfng* expression is that they are secondary to the regulation of Delta/Serrate genes that activate Notch. If proneural genes (or downstream transcription factors) regulate *lfng* directly, blocking Notch activation will lead to more cells expressing *lfng* due to the consequent loss of lateral inhibition and increase in neurogenesis. By contrast, if Notch activation regulates *lfng*, blocking Notch activation will decrease *lfng* expression. We therefore tested the effect of inhibiting Notch activation with DAPT (Geling et al., 2002), and found that this leads to increased *lfng* expression (Fig. 6A,C), concurrent with more neurogenesis marked by strong *deltaA* expression (Fig. 6B,D). A further possibility is that proneural genes and Notch activation synergize to upregulate *lfng*. However, we found no further change in *lfng* expression by combining triple proneural gene knockdown with the blocking of Notch activation with DAPT, or with the overexpression of *ngn1* together with dominant active Su(H) to activate the Notch pathway (data not shown). These data suggest that *lfng* is upregulated downstream of proneural genes, and not indirectly via Notch activation by Delta ligands.

**Lfng acts cell autonomously to inhibit neurogenesis**

The finding that *lfng* expression is upregulated downstream of proneural genes raises the question, which cells is *Lfng* required in? *Lfng* could act cell autonomously in progenitors to promote Notch activation and thus inhibit their differentiation. Alternatively, *lfng* expression in cells selected to differentiate could act non-autonomously to increase Notch activation in adjacent progenitors, as *Lfng* promotes the translocation of Delta to the cell surface (Sakamoto et al., 2002). These possibilities lead to different predictions for the effect of mosaic knockdown. In the former model, *lfng* knockdown cells will have increased differentiation, similar to the effect of inhibiting Notch pathway activation with dominant-negative CSL (DBM in Wettstein et al. (1997)). In the latter model, there would be no increase in the differentiation of *lfng* knockdown cells, as the increase in neurogenesis occurs in adjacent cells.

To test these models, we carried out transplantations to generate embryos mosaic for *lfng* MO plus GFP expression, and determined the relative number of *Lfng*-deficient cells that had differentiated or remained as progenitors in comparison with mosaic GFP expression alone. We found that there was increased differentiation of *lfng* MO cells transplanted into a noninjected host, as also occurred for cells overexpressing dn-CSL (Fig. 7A). Furthermore, in the reciprocal experiment there was decreased neuronal differentiation of wild-type cells that had been transplanted into a *lfng* MO-injected host (Fig. 7A). We observed that, when a large number of transplanted *lfng* MO cells were present, a lower proportion of these cells differentiated compared with embryos in which there were a low number of *Lfng*-deficient cells (Fig. 7B). These data are consistent with competition whereby *lfng* MO cells preferentially undergo neurogenesis at the expense of cells in which *Lfng* function is not inhibited. In mosaics with a low number of *Lfng*-deficient cells (Fig. 7D), most are competing with cells expressing *lfng*, and thus a high proportion of *Lfng*-deficient cells differentiate. By contrast, in mosaics with a large number of *Lfng*-deficient cells (Fig. 7E), many will be competing with each other and not be biased to differentiate preferentially at the expense of their neighbours.

**DISCUSSION**

The expression of *Lfng* initially in a segmental pattern and subsequently in neurogenic regions raised the possibility that this Notch modulator may have roles in the regulation of segmentation and/or neurogenesis. We find that *lfng* is expressed in neurogenic domains of the zebrafish hindbrain, where its expression occurs in progenitors and is rapidly downregulated in differentiating neurons. The results of loss-of-function studies reveal that *Lfng* is required to limit the amount of neurogenesis in the hindbrain and to maintain neural progenitors at late stages. Analysis of mosaic embryos reveals that *Lfng* acts cell autonomously in progenitors to inhibit their differentiation. Furthermore, the bias of *lfng* knockout cells to preferentially differentiate depends upon the degree of mosaicism, consistent with the competition of cells that occurs during the lateral inhibition of neurogenesis. Taken together, these findings suggest that *Lfng* acts within progenitor cells to promote the lateral inhibition of neurogenesis.

**Significance of segmental expression of *lfng***

In some tissues, Fringe homologues regulate boundary formation by modulating Notch activity at the interface of expressing and nonexpressing cells (Domínguez and de Celis, 1998; Laufer et al., 1997; Panin et al., 1997; Rodríguez-Esteban et al., 1997). It was therefore possible that the elevated expression of *lfng* in r2 and r4 reflected a role in hindbrain boundary formation. However, we found no effect of *lfng* knockdown on segmentation or boundary marker expression. Furthermore, hindbrain boundary cells initially form in *mib* mutants in which there is a major decrease in Notch activation, but are not maintained, as decreased lateral inhibition of neurogenesis leads to the loss of neural epithelial cells (Cheng et al., 2004). There is thus no evidence to support a role for Notch in hindbrain boundary formation. An alternative explanation for segmental *lfng* expression is suggested by the observation that the proneural gene *ascl1b* is initially expressed at higher levels.
throughout r2 and r4 (Amoyel et al., 2005), correlating with neurogenesis occurring in even- before odd-numbered segments (Bally-Cuif et al., 1998; Maves et al., 2002). The early segmental phase of lfng expression may therefore reflect the fact that neurogenesis is segmentally regulated, rather than suggest a role in segmentation.

**Notch in the lateral inhibition of neurogenesis**

There are similarities and differences between the effect of lfng knockdown and the major deficiency of Notch activation in mib mutants (Itoh et al., 2003; Jiang et al., 1996). In mib mutants, there is a 2- to 4-fold increase in early differentiating neurons, such as reticulospinal neurons, and the consequent depletion of progenitors leads to a decrease or loss of later-generated branchiomotor and commissural neurons, with neurogenesis almost absent by 24 hpf (Bingham et al., 2003; Jiang et al., 1996). lfng knockdown leads to a 1.7-fold increase in overall neurogenesis and increased production of many neuronal cell types, including branchiomotor neurons, somatic motor neurons, interneurons and commissural neurons. This is a milder neurogenic phenotype than mib mutants, consistent with Lfng increasing, rather than being essential for, Notch activation. As would be anticipated, lfng knockdown leads to later depletion of neural progenitors than in mib mutants, with normal expression of progenitor markers at 24 hpf and a decrease by 40 hpf. However, there is a distinct effect of the mib mutation compared with lfng knockdown on reticulospinal neurons, which are born during gastrulation and are the first to differentiate in the hindbrain (Hanneman et al., 1988; Mendelson, 1986). Whereas in mib mutants the single Mauthner reticulospinal neuron increases to 3-4 neurons (Jiang et al., 1996), there is no increase following lfng knockdown. One possibility is that the milder neurogenic effect of lfng knockdown cannot be detected for reticulospinal neurons due to their low number, or there may be differences in the regulation of their differentiation compared with subsequent neurogenesis.

Our findings appear contrary to a study in which retroviral-mediated overexpression of Lfng in the chick neural tube increases the number of neurons (de Bellard et al., 2007), as we found that transgenic expression of Lfng in r3/r5 decreases neurogenesis. One contributory factor is suggested by our observation that widespread knockdown of lfng has less of an effect on neurogenesis than occurs cell autonomously for mosaic knockdown, consistent with the competition to differentiate during the lateral inhibition of neurogenesis. This can explain our finding that widespread transgenic overexpression of Lfng within r3/r5 inhibits neurogenesis only to a modest extent. A potential explanation for increased neurogenesis in the chick neural tube is suggested by the major increase in cell proliferation that occurs following Lfng expression (de Bellard et al., 2007). Since effects on neuronal differentiation were analysed 24-48 hours after retroviral infection, expansion of the progenitor pool may underlie the increased number of neurons and outweigh a modest inhibition of differentiation by widespread Lfng expression. There would be less of an impact of such effects in our study, in which the number of neurons was analysed 6 hours after the onset of Gal4 driver expression.

**Notch1a and neuronal specification**

In addition to its role in mediating the lateral inhibition of neurogenesis, Notch mediates inhibitory or inductive signalling required for the specification of interneuronal subtype (Batista et al., 2008; Del Barrio et al., 2007; Peng et al., 2007). We find that in zebrafish, Notch1a is upregulated in differentiating neurons, suggesting that it is expressed downstream of proneural genes, whereas other Notch genes, including notch1b, are expressed predominantly in progenitors. These data suggest that there has been subfunctionalisation, as found for other genes duplicated in the zebrafish genome (Postlethwait et al., 2004), with expression of Notch1 divided between orthologues expressed in progenitors and neurons. We show Notch1a is required for interneuron subtype fate choice, but lfng knockdown leads to an increase of both interneuron subtypes, rather than a decrease in the population that requires Notch1a. We therefore conclude that Lfng acts to limit the amount of neuronal differentiation, but is not required for subtype specification regulated by Notch1a.

**Regulation of lfng expression during neurogenesis**

We find that lfng expression is decreased following knockdown of proneural genes, and increased following DAPT treatment that inhibits Notch activation, leading to increased proneural gene expression. Furthermore, the effect of proneural knockdown or overexpression on lfng expression was not exacerbated by the...
alteration of Notch activation, arguing against a synergistic input of proneural and Notch activity. These findings suggest that, like Delta genes, \textit{lfng} is a target of proneural proteins or downstream transcription factors and does not require Notch activity. This is consistent with studies in mouse suggesting that \textit{Lfng} is a direct target of proneural transcription factors (Castro et al., 2006), and furthermore, the proneural factor binding motif is present in the zebrafish \textit{lfng} gene (D. S. Castro, personal communication). However, whereas \textit{lfng} expression overlaps with \textit{deltaA} that is widely expressed in progenitors, it is not coexpressed with \textit{deltaB} in cells committed to neuronal differentiation and initiating migration from the neural epithelial layer. The rapid downregulation of \textit{lfng} upon the onset of neuronal differentiation suggests that there are regulatory inputs in addition to proneural genes, such as a transcription factor(s) restricted to progenitors.

Analysis of the effects of knockdown or overexpression of single proneural genes suggests that they have overlapping and specific roles in the regulation of \textit{lfng}. The finding that \textit{lfng} is upregulated by \textit{ngn1} overexpression but little affected by \textit{ngn1} knockdown suggests that other proneural genes also regulate \textit{lfng}. However, \textit{ascl1b} overexpression did not upregulate \textit{lfng}, and \textit{ascl1a} expression overlaps with \textit{lfng} only in dorsal regions. Further work is required to determine whether this complexity is due, for example, to other factors required for specific proneural genes to regulate \textit{lfng}.

\textbf{Lfng in the regulatory logic of the lateral inhibition of neurogenesis}

The initiation of neuronal differentiation requires an increase in proneural gene expression from the low level that occurs widely in the neural epithelium. This increase is enabled by positive feedback on proneural expression within cells (Bae et al., 2000; Culi and Modolell, 1998; Dubois et al., 1998; Koyano-Nakagawa et al., 2000), which is countered by inhibitory loops that occur within and between cells (Bai et al., 2007; Heitzler et al., 1996; Kunisch et al., 1994; Ohtsuka et al., 1999). The process of lateral inhibition requires that there is sufficient Notch activation in progenitors to inhibit proneural expression and thus prevent differentiation.

Our findings that \textit{lfng} is upregulated by proneural genes raises the question of where \textit{Lfng} acts in the regulatory logic of lateral inhibition. A potential clue is the observation that the level of proneural and Delta gene expression per cell appears to be higher following \textit{lfng} knockdown. This finding suggests that \textit{Lfng} acts in a feedback loop downstream of proneural genes, in which the promotion of Notch activation by \textit{Lfng} limits further upregulation of proneural and Delta genes in progenitors (Fig. 8). As found in other tissues, \textit{Lfng} may increase the binding of Notch to Delta expressed on adjacent cells, but it is not obvious in this model why \textit{lfng} would be upregulated in progenitors downstream of proneural genes. Such oscillations of proneural gene expression downstream of Delta Delta in the zebrafish hindbrain suggests that inhibition occurs by removal of the Notch-Delta complex from the cell surface (Matsuda and Chitnis, 2009).

Based upon these findings, we propose the following model (Fig. 8). Progenitor cells have widespread expression of proneural and downstream Delta genes, and compete to further upregulate proneural genes and laterally inhibit their neighbours. Due to cis-inhibition, there will inevitably be decreased activation of Notch in Delta-expressing cells. If cis-inhibition is too strong in progenitor cells, it will decrease their sensitivity to lateral inhibition, leading to positive feedback in which increased expression of proneural and Delta genes inhibits Notch activation that would normally inhibit further upregulation of proneural genes (Fig. 8). The coexpression of \textit{lfng} with Delta downstream of proneural genes prevents this loop by blocking cis-inhibition, thus preventing the inappropriate upregulation of proneural genes that would lead to neurogenesis. By contrast, in cells that have initiated differentiation, \textit{lfng} is downregulated and no longer coupled to Delta expression. Consequently, cis-inhibition of Notch by Delta will facilitate the further upregulation of proneural genes. This proposed change in cis-inhibition is consistent with recent work showing that in some progenitors DeltaD endocytosis is mainly due to trans-interactions, whereas in others cis-interactions are more important (Matsuda and Chitnis, 2009).

An alternative view of how \textit{Lfng} may regulate neurogenesis is suggested by evidence that oscillations of proneural gene expression maintain progenitor cells and sustained high levels of expression promote neurogenesis (Kageyama et al., 2008). Such oscillations require Notch activation in order to downregulate proneural expression from its peak level (Shimojo et al., 2008). A
desensitisation of Notch due to cis-inhibition by Delta would lead to decreased signalling, such that there is sustained proneural gene expression and consequently neurogenesis. Further insights into the role of Lfng may therefore be obtained by real-time visualisation of its expression, and by analysing whether Lfng knockdown affects oscillations of proneural gene expression.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/15/2523/DC1

References


Supplementary Figure 1

A

ATG  Exon1  Exon2  Intron1

Primers

P1  P3  P2

lfngEIII MO
Primers

B

DNA Ladder  0.56 pmol Ifng  0.56 pmol control  1.12 pmol Ifng  1.12 pmol control  Uninjected

1018 bp  506 bp

P1+P2: spliced mRNA (~370bp)
P1+P3: unspliced mRNA (~650bp)
Supplementary Figure 2

Control MO

Ifng^E177 MO

rfng  foxb1.2  krox20  ephrinB3

A  B  C  D

E  F  G  H
Supplementary Figure 3

<table>
<thead>
<tr>
<th>MO</th>
<th>ngn1</th>
<th>ascl1a</th>
<th>ascl1b</th>
<th>deltaA</th>
<th>deltaB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IfngE11</td>
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<table>
<thead>
<tr>
<th>MO</th>
<th>ngn1</th>
<th>ascl1a</th>
<th>ascl1b</th>
<th>deltaA</th>
<th>deltaB</th>
<th>her4</th>
<th>sox3</th>
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<tbody>
<tr>
<td>Control</td>
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Supplementary Figure 4

<table>
<thead>
<tr>
<th>Neurofilament</th>
<th>Zn-5</th>
<th>Zn-5</th>
<th>isl1</th>
<th>tbx20</th>
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</thead>
<tbody>
<tr>
<td><strong>Control MO</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
| A              | ![Control MO Neurofilament](image) A
| B              | ![Control MO Zn-5](image) B
| C              | ![Control MO Zn-5](image) C
| D              | ![Control MO isl1](image) D
| E              | ![Control MO tbx20](image) E |
| **Ifng** MO |
| F              | ![Ifng MO Neurofilament](image) F
| G              | ![Ifng MO Zn-5](image) G
| H              | ![Ifng MO Zn-5](image) H
| I              | ![Ifng MO isl1](image) I
| J              | ![Ifng MO tbx20](image) J |

<table>
<thead>
<tr>
<th>pax2.1</th>
<th>gad67</th>
<th>evx1</th>
<th>lhx2</th>
<th>lhx9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control MO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| ![Control MO pax2.1](image) K
| ![Control MO gad67](image) L
| ![Control MO evx1](image) M
| ![Control MO lhx2](image) N
| ![Control MO lhx9](image) O |
| **Ifng** MO |
| ![Ifng MO pax2.1](image) P
| ![Ifng MO gad67](image) Q
| ![Ifng MO evx1](image) R
| ![Ifng MO lhx2](image) S
| ![Ifng MO lhx9](image) T |
Supplementary Figure 5

<table>
<thead>
<tr>
<th></th>
<th>18 hpf</th>
<th>24 hpf</th>
<th>34 hpf</th>
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<tbody>
<tr>
<td>notch1a</td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>r2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r4</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

| notch1b | D      | E      | F      |
|         |        |        |        |

| notch3   | G      | H      | I      |
|          |        |        |        |

| notch1a deltaA | J      |         |
|                | r3     | r4      |
| notch1a deltaB | K      |         |
|                | r3     | r4      |

18 hpf, 24 hpf, 34 hpf