Development 137, 437-445 (2010) doi:10.1242/dev.036806

Domain-specific control of neurogenesis achieved through patterned regulation of Notch ligand expression

Ulrika Marklund[1], Emil M. Hansson[1], Erik Sundström[2], Martin Hrabé de Angelis[3,4], Gerhard K. H. Przemeck[3], Urban Lendahl[1,*], Jonas Muhr[1,5,*] and Johan Ericson[1,*]

SUMMARY

Homeodomain (HD) transcription factors and components of the Notch pathway [Delta1 (Dll1), Jagged1 (Jag1) and the Fringe (Fng) proteins] are expressed in distinct progenitor domains along the dorsoventral (DV) axis of the developing spinal cord. However, the internal relationship between these two regulatory pathways has not been established. In this report we show that HD proteins act upstream of Notch signalling. Thus, HD proteins control the spatial distribution of Notch ligands and Fng proteins, whereas perturbation of the Notch pathway does not affect the regional expression of HD proteins. Loss of Dll1 or Jag1 leads to a domain-specific increase of neuronal differentiation but does not affect the establishment of progenitor domain boundaries. Moreover, gain-of-function experiments indicate that the ability of Dll1 and Jag1 to activate Notch is limited to progenitors endogenously expressing the respective ligand. Fng proteins enhance Dll1-activated Notch signalling and block Notch activation mediated by Jag1. This finding, combined with the overlapping expression of Fng with Dll1 but not with Jag1, is likely to explain the domain-specific activity of the Notch ligands. This outcome is opposite to the local regulation of Notch activity in most other systems, including the *Drosophila* wing, where Fng co-localizes with Jagged/Serrate rather than Dll/Delta, which facilitates Notch signalling at regional boundaries instead of within domains. The regulation of Notch activation in the spinal cord therefore appears to endow specific progenitor populations with a domain-wide autonomy in the control of neurogenesis and prevents any inadequate activation of Notch across progenitor domain boundaries.

KEY WORDS: CNS, Homeodomain, Neural patterning, Neurogenesis, Notch pathway

INTRODUCTION

The establishment of the central nervous system (CNS) depends on the generation of functionally distinct neurons produced in appropriate numbers and at correct positions. In the developing spinal cord, distinct classes of neurons are produced at specific locations along the dorsoventral (DV) axis (Jessell, 2000; Poh et al., 2002). These neurons include motoneurons (MNs) and various types of interneurons, which originate from progenitor cells organized in specific domains along the DV axis. Genetic gain- and loss-of-function experiments have revealed that the establishment of DV domains, and neurogenesis within these domains, is influenced by homeodomain (HD) transcription factors, proneural basic helix-loop-helix (bHLH) proteins and the Notch signalling pathway. However, the internal relationship between these sets of regulatory pathways is currently incompletely understood.

HD transcription factors are expressed in distinct patterns along the DV axis in the developing neural tube. Their expression is confined to neural progenitors and is controlled by a ventral-to-dorsal graded activity of Sonic Hedgehog signalling (Jessell, 2000). The combinatorial expression of these HD proteins subdivides the spinal cord into a number of distinct progenitor domains, which each gives rise to specific subtypes of postmitotic neurons (Jessell, 2000). Cross-repressive interactions between complementary pairs of HD proteins further refine and sharpen the boundaries of these progenitor domains (Briscoe et al., 2000; Muhr et al., 2001). For instance, cross-repression between the HD transcription factor Dbx2 and Nkx6.1 leads to the establishment of a sharp progenitor domain boundary between the p2 and p1 domains (Briscoe et al., 2000; Sander et al., 2000; Vallstedt et al., 2001). Genetic loss- and gain-of-function experiments have revealed the importance of HD proteins for the specification of neuronal identities (Briscoe et al., 2000; Jessell, 2000; Pierani et al., 2001). However, the precision by which neuronal subtypes are generated during CNS development implies that mechanisms controlling cell type and cell number must be stringently coordinated. Indeed, the transcription factors Olig2 and Lmx1a not only determine the fate of somatic MNs and midbrain dopamine neurons, respectively, but also influence the rate of neurogenesis by promoting expression of the proneural bHLH protein Ngn2 in ventral progenitor cells (Andersson et al., 2006; Dubreuil et al., 2002; Lee et al., 2005; Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2003; Scardigli et al., 2001; Sugimori et al., 2007). Thus, one regulatory means to control neuronal subtype identities and cell numbers appears to involve a regulatory interaction between cell fate-determining transcription factors and proneural bHLH proteins (Scardigli et al., 2003).

Like HD proteins, components of the Notch pathway (Dll1, Jag1 and Fng proteins) are expressed in distinct patterns along the DV axis in the developing spinal cord (Henrique et al., 1995; Johnston et al., 1997; Lindsell et al., 1996; Myat et al., 1996; Sakamoto et al., 1997). Notch signalling is a cell-cell communication system and

[1]Department of Cell and Molecular Biology, Karolinska Institutet, SE-171 77 Stockholm, Sweden. [2]Division of Neurodegeneration, Department of Neurobiology, Care Sciences and Society, Novum, Karolinska Institutet, SE-141 86 Stockholm, Sweden. [3]Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Institute of Experimental Genetics, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany. [4]Technische Universität München, Center for Life and Food Science, Chair of Experimental Genetics, Weihenstephan, Alte Akademie 8, 85354 Freising, Germany. [5]Ludwig Institute for Cancer Research, Karolinska Institute, Box 240, SE-171 77 Stockholm, Sweden.

*Authors for correspondence (urban.lendahl@ki.se; jonas.muhr@licr.ki.se; johan.ericson@ki.se)

Accepted 27 November 2009

activation of Notch signalling results from the interaction between the Notch receptor and its ligands, Delta and Jagged, expressed by adjacent cells. The ligand receptor interaction induces a proteolytic cleavage of the Notch protein and nuclear translocation of its intracellular domain (Notch ICD), which interacts with the DNA-binding protein CSL (RBP-Jk) to activate Notch downstream genes, such as the Hey and Hes genes. These proteins have been demonstrated to, at least in part, mediate their functions by repressing proneural proteins, such as Ngn2 (Louvi and Artavanis-Tsakonas, 2006). Also, Notch receptors are modulated by the Fringe glycosyltranserases, which extend *O*-glycosylation carried out by the *O*-fucosyltransferases (O-fut1 in *Drosophila* and Pofut1 in mammals) (Stanley, 2007). Fringe-mediated glycosylation modulates the ability of the Notch receptor to respond to the ligands. In many situations, Fng enhances Dll1-Notch interactions while reducing Jag1-Notch interactions (Hicks et al., 2000; Moloney et al., 2000; Shimizu et al., 2001; Xu et al., 2007). Despite these findings, we do not understand the roles and functional relationship of Notch ligands and Fng proteins in regulating neurogenesis and neuronal subtype specification along the DV axis of the neural tube. Moreover, the functional relevance of their patterned distribution along the DV axis of the neural tube has not been resolved.

Given that both HD proteins and Notch signalling are important for the control of CNS development, and that both exhibit distinct expression domains in the developing spinal cord, we have in this report addressed the relationship between HD proteins and Notch signalling. We provide evidence that HD proteins regulate the spatial distribution of Notch ligands and Fng proteins in distinct DV progenitor domains. Furthermore, we demonstrate that loss of Dll1 or Jag1 signalling leads to a domain-specific increase of neuronal differentiation but does not affect the establishment of progenitor domain boundaries. Our data also suggest that signalling between Dll1$^+$ and Jag1$^+$ domains is prohibited along the DV axis of the neural tube, which is in contrast to the situation in zebrafish rhombomeres and the *Drosophila* wing, where Notch signalling is enhanced at boundaries between domains (Wu and Rao, 1999). We discuss a model in which the different configurations of Fng expression with regard to ligand expression may explain the observed differences in the outcome of Notch signalling in different tissues. Moreover, we show how this expression pattern might allow for quantitative different levels of Notch signalling in distinct progenitor domains, as well as prevent inadequate activation of Notch signalling across boundaries between adjacent progenitor cell populations. Thus, this mechanism provides a means to individually control the numbers of specific neurons produced by neighbouring progenitor domains along the DV axis of the neural tube.

MATERIALS AND METHODS

Expression constructs and in ovo electroporation

cDNA encoding chick Nkx6.1 and mouse Nkx2.2 were misexpressed using the retroviral vector RCASBP(B), and mouse *Dll1*, *Jag1*, *Dbx1* and *Mfng* and chick *Olig2* were misexpressed using the CMVβ/actin-based pCAGGS vector. DNA constructs were electroporated into the neural tube of Hamburger-Hamilton (HH) stage 10-11 chick embryos (Briscoe et al., 2000). Embryos were harvested and fixed 30-48 hours post-transfection (hpt) and processed for immunohistochemistry or in situ hybridization.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as previously described (Briscoe et al., 2000). The following antibodies were used: rat anti-Jag1 (Developmental Studies Hybridoma Bank), rat anti-PDGRα (BD Pharmingen); mouse anti-Pax7, anti-Nkx2.2, anti-Evx1/2 and anti-En1 (Developmental Studies Hybridoma Bank), mouse anti-GATA3 (Santa Cruz, sc9009), mouse anti-Ngn2 (kind gift from D. Anderson, California Institute of Technology, CA, USA) mouse anti-HuC/D (Molecular Probes), mouse anti-Brn3a (Chemicon); rabbit anti-cleaved caspase-3 (Cell Signaling), rabbit anti-Dbx1 and anti-Dbx2 (kind gift from A. Pierani, Institut Jacques Monod, Paris, France), rabbit anti-Pax2 (Nordic Biosite), rabbit anti-Nkx6.1 (Briscoe et al., 2000), rabbit anti-Jag1 (Santa Cruz), rabbit anti-Gsh1/2 (kind gift from M. Goulding, The Salk Institute, CA, USA), rabbit anti-Chx10 (Ericson et al., 1997), rabbit anti-Dll1 (Alexis Biochemicals), rabbit anti-Nestin (BD Biosciences Pharmingen), rabbit anti-Sox3 (kind gift from T. Edlund, Umeå University, Sweden); guinea pig anti-Isl1/2 (Tsuchida et al., 1994), guinea pig anti-Olig2 (kind gift from T. Jessell, Colombia University, NY, USA); goat anti-β-gal (Biogenesis), goat anti-Sox10 (Santa Cruz, N20). In situ hybridization was performed essentially as described (Tsuchida et al., 1994), using probes for mouse *Jag1* and *Dll1* (Lindsell et al., 1996), *Lfng* (IMAGE4000728), *Mfng* (IMAGE575363), *Hes5* (Akazawa et al., 1992), *Dbx1* (Invitrogen IRAVp968a07126d) and *Olig2* (kind gift from B. Novitch, University of Michigan, MI, USA), and chick *Jag1* and *Delta1* (isolated from cDNA libary), *Lfng* (kind gift from C. Tabin, Harvard Medical School, MA, USA), *Mfng* (Geneservice ChEST679I3), *Nkx6.1* (Briscoe et al., 2000), *Dbx1* and *Dbx2* (Pierani et al., 2001), and *Gsh1* (Geneservice ChEST1010i7).

Mouse mutants and human embryos

The generation of Dbx1 and Nkx6.1 mutant mice was previously reported (Pierani et al., 2001; Sander et al., 2000). Jagged1$^{Ndr/Ndr}$ (Nodder) mice were identified in an ENU screen at Ingenium, Germany. These mice carry a single base-pair mutation in the extracellular part of jagged 1. The name Nodder reflects the nodding behaviour and balance defects in the heterozygous state. Mice carrying the *Dll1^{lacZ}* knock-in allele have been described previously (Hrabe de Angelis et al., 1997). Human embryos (5.5 weeks of gestation) were collected after elective routine abortions with consent given by the pregnant woman. The tissue collection was approved by the Regional Human Ethics Committee, Stockholm.

Statistical analysis

Student's *t*-test comparisons were performed for all statistical examinations. Bars indicate means±s.d. (standard deviation). Significance for the tests were assumed at the level of $P<0.05$, $n=4$ ($*P<0.05$, $0.01>**P>0.001$, $***P<0.001$).

RESULTS

Delta1 and Jagged1 expression is confined to specific progenitor domains generating distinct neuronal subtypes

To examine the role of Dll1 and Jag1 in the developing spinal cord, we applied in situ hybridization and immunohistochemistry to map the regional expression of *Dll1*, *Jag1*, Lunatic Fringe (*Lfng*) and Manic Fringe (*Mfng*) in relation to HD proteins defining different DV progenitor domains (Jessell, 2000; Poh et al., 2002). In HH stage 20 chick embryos, *Dll1*, *Lfng* and *Mfng* had similar expression patterns: the genes were broadly expressed in the ventral and dorsal thirds of the neural tube, and in a narrow intermediate stripe (Fig. 1A,B,D; data not shown) (Johnston et al., 1997; Lindsell et al., 1996). The ventral expression of *Dll1*, *Lfng* and *Mfng* coincided precisely with the expression domain of *Nkx6.1* (Fig. 1E; data not shown), the intermediate stripe correlated to *Dbx1* expression, whereas the dorsal expression mapped to the *Gsh1/Gsh2* expression domain (Fig. 1G,H; data not shown). Jag1 expression was primarily confined to two narrow domains in the intermediate part of the spinal cord, where it was complementary to the expression of Dll1 (Fig. 1B,C,Q) (Lindsell et al., 1996). These *Jag1*$^+$ cells correlated with the expression domain of *Dbx2* but not *Dbx1*, *Nkx6.1* or *Gsh1/Gsh2* (Fig. 1E-H). Similar expression patterns were observed in the embryonic

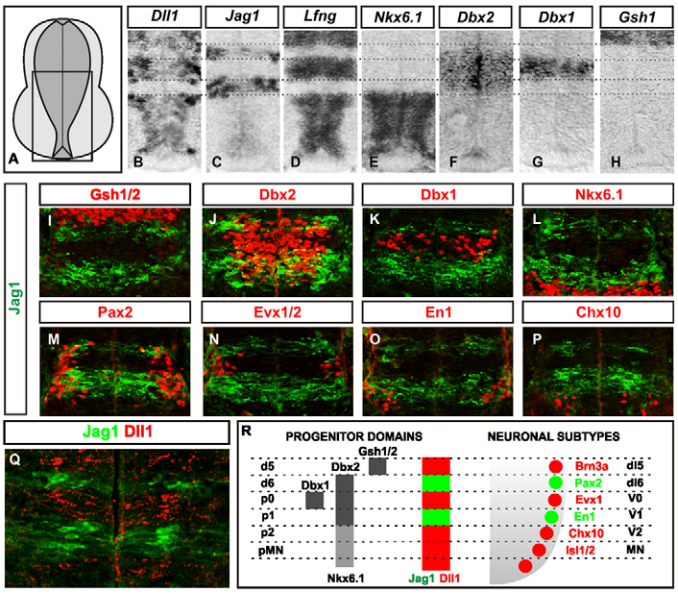

Fig. 1. Notch ligand expression correlates to distinct progenitor domains. (**A**) Schematic of a transverse neural tube section indicating the region analysed in B-H. (**B-H**) Localization of mRNA for *Dll1*, *Jag1* and *Lfng* in comparison with HD transcription factors in transverse chick spinal cord sections at embryonic day 3.5. (**I-P**) Jag1 protein expression in relation to the expression of the indicated HD transcription factors and neuronal subtype-specific markers in E10.5-E11 mouse spinal cords. The intermediate part of the spinal cord was analyzed. (**Q**) Immunohistochemistry demonstrating the complementary expression of Dll1 and Jag1 in embryonic day 3.5 chick spinal cord. (**R**) Schematic showing the expression of Jag1 and Dll1 in relation to progenitor domains in the developing chick spinal cord.

chick, mouse and human spinal cord (Fig. 1I-L; see also Fig. S1A-E,K,L in the supplementary material). Thus, the expression of *Jag1*, *Dll1*, *Lfng* and *Mfng* correlates precisely with defined DV progenitor domains (Fig. 1R), and this expression pattern appears to be conserved in higher vertebrates.

Different DV progenitor domains generate distinct neuronal subtypes that can be distinguished based on their expression of molecular markers (Helms and Johnson, 2003; Poh et al., 2002). Expression of Jag1 in cells with a Dbx2^{+}/Dbx1^{-} identity (Fig. 1J,K) indicate that dorsal Jag1^{+} cells correspond to d6 progenitors generating dI6 neurons, whereas the ventral stripe delineates p1 progenitors giving rise to V1 neurons. In support for this, we could at embryonic day (E)10.5 in the mouse, detect dorsal Jag1^{+} cells that co-expressed Pax2 [expressed in all neurons derived from Dbx2^{+} cells (Matise and Joyner, 1997)] but not the dI5 marker Brn3a or the V0 marker Evx1/Evx2 (Fig. 1M,N; data not shown) (Pierani et al., 1999). Ventral Jag1^{+} cells co-expressed the V1 marker En1, but not markers for juxtaposed V0, V2a or V2b neurons (Evx1/Evx2, Chx10 or Gata3) (Fig. 1N-P; data not shown) (Matise and Joyner, 1997). Similar expression of Jag1 was detected also in the chick and human neural tube (see Fig. S1F-J,M-O in the supplementary material; data not shown). Thus, Jag1 is expressed in Dbx2^{+}/Dbx1^{-} progenitors generating V1 and dI6 neurons, whereas expression of Dll1, *Lfng* and *Mfng* is confined to Dbx2^{+}/Dbx1^{+} p0 progenitors generating V0 neurons and to ventral Nkx6.1^{+} progenitors producing V2 neurons and MNs.

The regional expression of Notch ligands is controlled by HD proteins

The precise correlation between the expression of *Dll1*, *Jag1* and Fng genes and specific DV progenitor domains implies that these genes might be regulated by HD proteins involved in DV pattern formation. However, because Notch signalling has been shown to influence boundary formation and the affinity properties of cells in certain tissues (Baek et al., 2006; Herranz and Milan, 2006), it is also possible that the Notch pathway has a reciprocal input on DV patterning.

To examine this, we analysed the expression of *Jag1*, *Dll1*, *Lfng* and *Mfng* in Nkx6.1 and Dbx1 loss- and gain-of-function experiments. We focused on these HD proteins because they confer functional identity to p2 and p0 progenitors expressing Dll1 and concomitantly suppress the establishment of p1 progenitors expressing Jag1 (Briscoe et al., 2000; Pierani et al., 2001; Sander et al., 2000). Also, repressive interactions between Nkx6 and Dbx class proteins are known to be essential for the establishment of p0, p1 and p2 progenitor domain boundaries (Briscoe et al., 2000; Muhr et al., 2001; Vallstedt et al., 2001). In chick in ovo electroporation experiments, forced expression of Nkx6.1 extinguished *Dbx1*, *Dbx2* and *Jag1* expression (Fig. 2A,B; data not shown) (Briscoe et al., 2000), and induced ectopic expression of *Dll1*, *Lfng* and *Mfng*, resulting in an uninterrupted expression of these genes in the intermediate neural tube (Fig. 2C,D; see also Fig. S2A in the supplementary material). Conversely, in *Nkx6.1*$^{-/-}$ embryos at E10.5 (Sander et al., 2000), the *Jag1*$^{+}$ domain was ventrally expanded into the p2 and pMN domains (Fig. 2I,J; Fig. S3 in the supplementary material), and the expression of *Dll1*, *Lfng* and *Mfng* was reduced (Fig. 2K-N; Fig. S2C,D in the supplementary material). However, *Jag1* expression did not extend to the ventral midline, and expression of *Dll1* and *Lfng* could still be detected in Nkx2.2^{+} p3 progenitors in Nkx6.1 mutants (Fig. 2I-N; see also Fig. S3 in the supplementary material), implying that factors in addition to Nkx6.1 influence expression of Notch ligands and Fng proteins in the ventral neural tube. Consistent with this, Ngn2 expressed in the pMN domain has been shown to directly regulate Dll1 expression (Castro et al., 2006), and we found that Nkx2.2 (see Fig. S3 in the supplementary material) and Olig2 (Novitch et al., 2001) could downregulate Jag1 expression when overexpressed in intermediate positions of the neural tube (data not shown).

Forced expression of Dbx1 did not affect the expression of Dbx2 (data not shown), but repressed the dorsal and ventral stripes of *Jag1* expression (Fig. 2E,F). Also, similar to experiments with Nkx6.1 overexpression, the suppression of *Jag1* was followed by a continuous expression of *Dll1*, *Lfng* and *Mfng* in the intermediate neural tube (Fig. 2G,H; Fig. S2B in the supplementary material). In *Dbx1*$^{-/-}$ embryos (Pierani et al., 2001), by contrast, the intermediate stripe of *Dll1*, *Mfng* and *Lfng* expression was lost (Fig. 2Q-T; Fig. S2E,F in the supplementary material), and the expression of *Jag1* encompassed the entire *Dbx2*$^{+}$ domain (Fig. 2O,P). Together, these data establish that Nkx6.1 and Dbx1 control the DV expression profile of Notch ligands by suppressing the expression of Jag1 and promoting that of Dll1.

We next examined DV patterning in mice carrying loss-of-function mutations for Dll1 (Hrabe de Angelis et al., 1997) and Jag1 (Jag1$^{Ndr/Ndr}$; E.M.H. and U.L., unpublished). Apart from a slight enlargement of the floor plate in *Dll1*$^{-/-}$ mutant embryos (Przemeck et al., 2003), the initial establishment of DV progenitor domains appeared unaffected in Dll1 and Jag1 mutants, as indicated by an unaffected expression patterning of HD proteins (Fig. 2Y) and a normal degree of intermingling of cells at progenitor domain

boundaries (Fig. 2U,V,Z). Also, there was no change in the patterned expression of Jag1 in *Dll1*$^{-/-}$ or of Dll1 in *Jag1*$^{Ndr/Ndr}$ embryos (Fig. 2W,X; data not shown). Moreover, misexpression of Dll1 or Jag1 in the chick neural tube did not alter the regional expression of *Nkx6.1*, *Dbx1*, *Dbx2*, *Dll1* or *Jag1* (see Fig. S4A-L in the supplementary material). Together, these data show that the positional expression of *Dll1*, *Jag1*, *Lfng* and *Mfng* is regulated by Nkx6.1 and Dbx1, while neither Dll1 nor Jag1 have any obvious reciprocal effect on DV pattern formation.

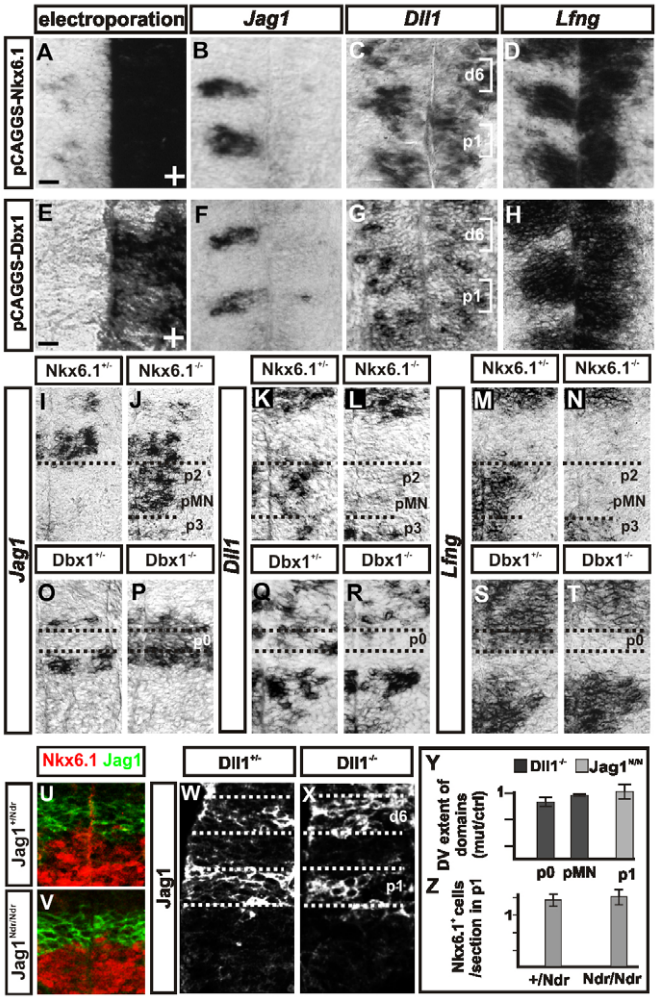

Fig. 2. HD transcription factors regulate the patterned expression of Notch regulatory proteins. (**A-H**) Misexpression of Nkx6.1 or Dbx1 for 42 hours in the chick neural tube suppressed the expression of *Jag1* (B,F) but upregulated the expression of *Dll1* and *Lfng* in the p1 and d6 domain (C,D,G,H). (**I-N**) In E10.5 *Nkx6.1*$^{-/-}$ mouse embryos, *Jag1* expression was ventrally expanded, replacing the expression of *Dll1* and *Lfng*. (**O-T**) In E10.5 *Dbx1*$^{-/-}$ embryos, the expression of *Dll1* and *Lfng* was repressed from the p0 domain, whereas *Jag1* expression was upregulated in the corresponding domain. (**U**,**V**,**Z**) The integrity of the border between the Nkx6.1/Dll1 and Dbx2/Jag1 domains was maintained in *Jag1*$^{Ndr/Ndr}$ mutants, as assessed by the numbers of Nkx6.1^{+} cells in the p1 domain in *Jag1*$^{+/Ndr}$ and *Jag1*$^{Ndr/Ndr}$ embryos (*n*=3, Z). (**W**,**X**) The expression domains of Jag1 remained unchanged in Dll1 mutant neural tubes. (**Y**) The DV extent of HD proteins was essentially unaffected in *Dll1*$^{-/-}$ and *Jag1*$^{Ndr/Ndr}$ embryos. The numbers of cells along the DV axis in the p1 domain was estimated by the number of Nkx6.2^{+} cells in E10.5 *Jag1*$^{+/Ndr}$ and *Jag1*$^{Ndr/Ndr}$ embryos. The extent of the p0 and pMN domains in *Dll1*$^{+/-}$ and *Dll1*$^{-/-}$ embryos was measured by the number of cells between the two Jag1^{+} domains (E10.5) and the number of Olig2^{+} cells (E9.5), respectively (*n*=3). The values were normalized to the decreased DV length of the *Dll1*$^{-/-}$ spinal cord.

Dll1 and Jag1 exert domain-specific control of neural differentiation

The normal establishment of DV progenitor domains in *Dll1*$^{-/-}$ and *Jag1*$^{Ndr/Ndr}$ mutants prompted us to examine the generation of specific neuronal subtypes in these mice. We quantified the generation of neuronal subtypes derived from Dll1^{+} or Jag1^{+} progenitors, and restricted our analysis to the intermediate and ventral neural tube. In *Dll1*$^{-/-}$ embryos, there was a significant overproduction of Isl1/Isl2^{+} MNs at E9.5 (Fig. 3E,E′,K), and an increased number of Chx10^{+} V2a, Gata3^{+} V2b and Evx1/Evx2^{+} V0 neurons at E10.5 (Fig. 3A,A′,C,C′,D,D′,K,Z). The generation of V1 neurons, however, was unaffected, as indicated by similar numbers of En1^{+} cells in Dll1 mutants and littermate controls at E10.5 (Fig. 3B,B′,K,Z). By contrast, in Jag1 mutants at E10.5, there was an approximately twofold increase of En1^{+} V1 neurons, whereas the production of MNs, V2a, V2b and V0 neurons was similar to that in controls (Fig. 3F-J′,K,Z). A similar region-specific effect on neurogenesis was observed also in the brainstem of *Dll1*$^{-/-}$ and *Jag1*$^{Ndr/Ndr}$ embryos (data not shown). In summary, these data reveal that Dll1 and Jag1 control neurogenesis in a progenitor domain-specific fashion along the DV axis of the neural tube.

We next examined the expression of the Notch target gene *Hes5* (Kageyama and Nakanishi, 1997) and the proneural bHLH protein Ngn2 (Bertrand et al., 2002) in the p0– and p1 domains in *Dll1*$^{-/-}$ and *Jag1*$^{Ndr/Ndr}$ mutants between E9.0 and E10.5. The expression of Dbx1 or Jag1 was used as a positional landmark for the p0– and p1 domains in these experiments (data not shown). In line with the overproduction of Evx1/Evx2^{+} V0 neurons, there was a significant loss of *Hes5* expression within the p0 domain in Dll1 mutants at E10.5, whereas *Hes5* expression in adjacent Jag1^{+} p1 progenitors was similar to that in control embryos (Fig. 3O,P). The reduction of *Hes5*$^{+}$ cells in the p0 domain was accompanied by a local increase in the number of Ngn2^{+} cells (Fig. 3L,M,Y). In *Jag1*$^{Ndr/Ndr}$ mutants, the p0 domain appeared normal and there was instead reduced numbers of *Hes5*$^{+}$ cells and an increase of Ngn2^{+} cells in the p1 domain at E10.5 (Fig. 3O,Q,L,N,Y). Also the number of cells expressing the neural progenitor marker Sox3 was reduced by ~40-50% in the p0 domain of *Dll1*$^{-/-}$ mice, and a corresponding reduction was observed in the p1 domain of *Jag1*$^{Ndr/Ndr}$ mutants (Fig. 3Y), suggesting that the regional overproduction of neurons in these mice occurs at the expense of undifferentiated progenitor cells. At early embryonic stages (E9-E9.5), however, we did not detect any significant difference in *Hes5* or Ngn2 expression in *Dll1*$^{-/-}$ or *Jag1*$^{Ndr/Ndr}$ mutants compared with in littermate heterozygous controls (Fig. 3R-T; data not shown). Moreover, there was no obvious premature induction of neurogenesis in *Dll1*$^{-/-}$ or *Jag1*$^{Ndr/Ndr}$ mutants, as indicated by the absence of En1^{+} and Evx1/Evx2^{+} neurons at E9.5 (Fig. 3U-X). Together, these data suggest that the overproduction of neurons in Dll1 and Jag1 mutants reflect an increased pace, rather than a premature onset, of neurogenesis.

Notch signalling has also been implicated in the control of gliogenesis (Rowitch, 2004; Taylor et al., 2007). Dll1 and Jag1 mutant embryos die at E12, which precluded any extensive analysis of gliogenesis in these mice. Nevertheless, as indicated by the lack of Olig2, Pdgfrα and Sox10 expression at E11.5 (see Fig. S5C-H in the supplementary material), there was no premature specification of oligodendrocyte precursors (OLPs) in Dll1 mutants. Instead, the

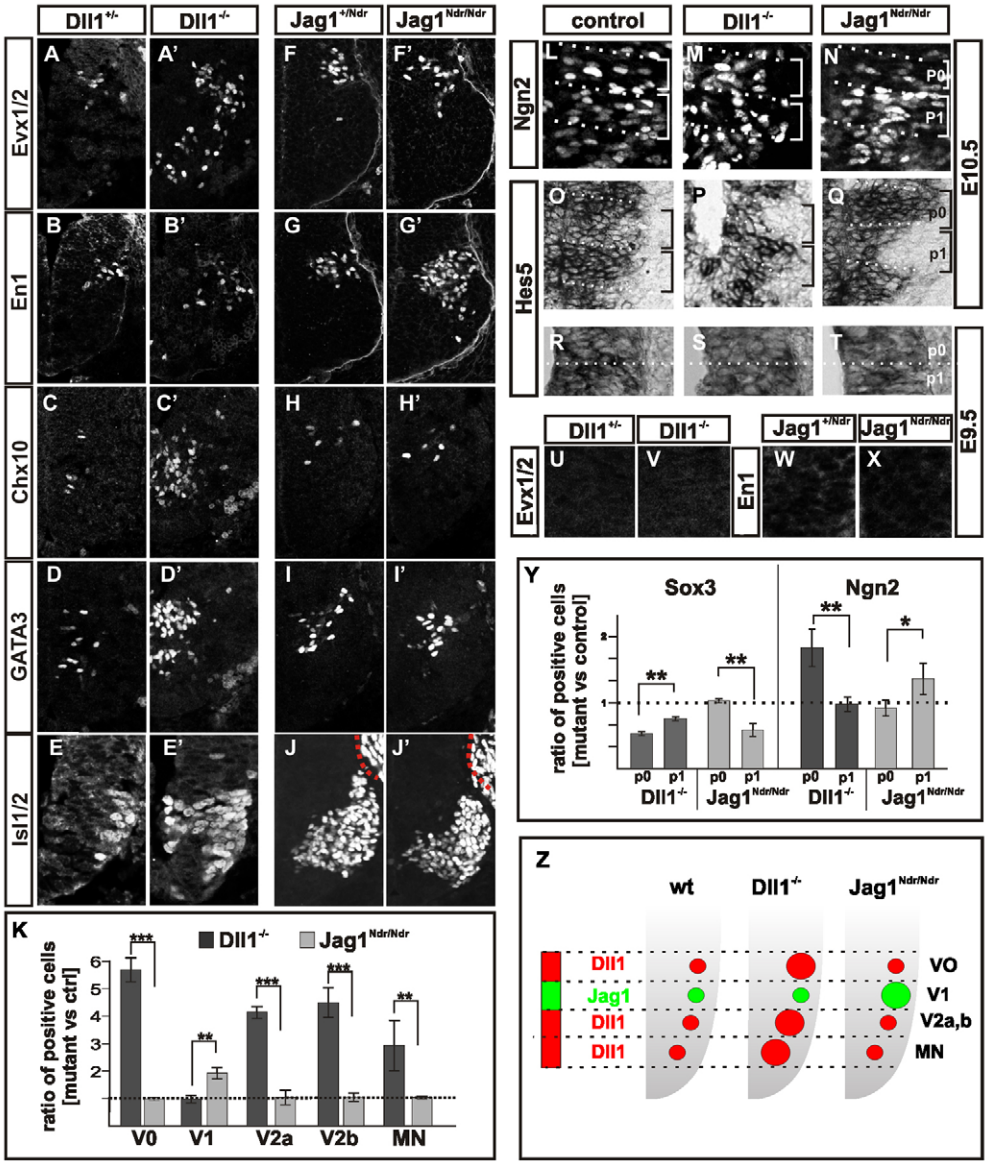

Fig. 3. Domain-specific effects on neurogenesis in *Jag1*$^{Ndr/Ndr}$ and *Dll1*$^{-/-}$ mouse mutants. (**A-E'**) Characterization of neuronal subtypes in *Dll1*$^{+/-}$ and *Dll1*$^{-/-}$ spinal cords analyzed at E10.5 (A-D') or E9.5 (E,E'). (**F-J'**) Examination of neuronal subtypes in *Jag1*$^{+/Ndr}$ and *Jag1*$^{Ndr/Ndr}$ spinal cords analyzed at E10.5. (**K**) Statistical analysis of experiments shown in (A-J'; *n*=4). (**L-N**) Ngn2 expression was upregulated in the p0 and p1 domain of *Dll1*$^{-/-}$ (L,M,Y) and *Jag1*$^{Ndr/Ndr}$ (L,N,Y) spinal cords, respectively (*n*=3). (**O-Q**) At E10.5, expression of *Hes5* was downregulated in the p0 and p1 domain of *Dll1*$^{-/-}$ (P) and *Jag1*$^{Ndr/Ndr}$ (Q) spinal cords, respectively, compared with in control spinal cords (O). Brackets outline progenitor domains and dashed lines boundaries in L-Q. (**R-T**)At E9.5, no decrease in *Hes5* expression could be detected in either *Jag1*$^{Ndr/Ndr}$ or *Dll1*$^{-/-}$ spinal cords. (**U-X**) At E9.5, no premature generation of Evx1/2^{+} V0 neurons (U,V) or En1^{+} V1 neurons (W,X) could be detected in Dll1 or Jag1 mutant spinal cords. (**Y**) The number of Sox3^{+} and Ngn2^{+} cells in the p0 and the p1 domain in *Dll1*$^{-/-}$ and *Jag1*$^{Ndr/Ndr}$ E10.5 spinal cords (*n*=3). (**Z**) Schematic showing the effects on neuronal subtypes in *Jag1*$^{Ndr/Ndr}$ and *Dll1*$^{-/-}$ spinal cords.

extensive loss of Olig2^{+} progenitors observed in Dll1 mutants after E10.5 (see Fig. S5A-D in the supplementary material) indicates a progressive depletion of pMN progenitors over time, and implies that Dll1 function is required to sustain a pool of presumptive MN/OLP progenitors at late developmental stages.

Progenitor cell responsiveness to Dll1 and Jag1 is patterned along the DV axis of the neural tube

Glycosylation of Notch receptors by Fng proteins has been shown in some situations to facilitate Dll1 signalling and constrain the ability of Jag1 to activate the Notch receptor (Stanley, 2007), whereas, for example, in the somites Lfng appears instead to inhibit Dll1-Notch signalling (Dale et al., 2003). To examine how Fng proteins affected Dll1 and Jag1 signalling in the developing spinal cord, we ectopically expressed Mfng, Dll1 or Jag1 in chick by in ovo electroporation and analyzed the generation of neuronal subtypes derived from progenitor domains endogenously expressing Dll1 or Jag1. Overexpression of Mfng resulted in a ~10-20% reduction of Isl1/Isl2^{+} MNs and Evx1/Evx2^{+} V0 neurons 40 hours after electroporation (Fig. 4A,C,D). The number of V1 neurons, however, was instead increased by ~1.6-fold in response to Mfng expression (Fig. 4B,D). These data suggest that

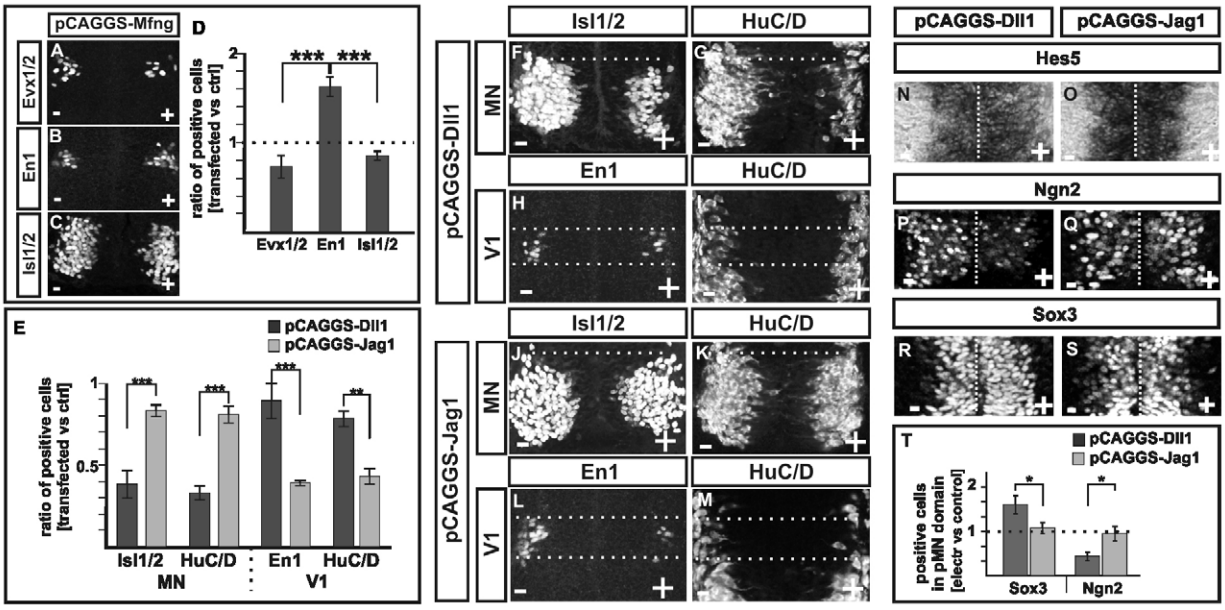

Fig. 4. Domain-specific response to Dll1 and Jag1 by regional expression of Fng. (**A-D**) Misexpression of Mfng for 40 hours in the chick neural tube decreased the number of Evx1/Evx2$^+$ V0 interneurons and Isl1/Isl2$^+$ MNs (A,C,D), whereas the number of En1$^+$ V1 interneurons was increased (B,D; *n*=4). (**E-I**) Misexpression of Dll1 for 45-48 hours decreased the number of Isl1/Isl2$^+$ and HuC/D$^+$ neurons derived from the pMN domain (E-G), whereas Dll1 only had a small effect on neurons derived from the p1 domain (E,H,I; *n*=3-4). (**J-M**) Electroporation of *Jag1* for 45-48 hours decreased the number of En1$^+$ and HuC/D$^+$ neurons derived from the p1 domain (E,L,M), whereas Jag1 only had a small effect on the number of Isl1/Isl2$^+$ or HuC/D$^+$ cells derived from pMNs (E,J,K; *n*=3-4). (**N-T**) Dll1 misexpression increased the number of Hes5$^+$ (N) and Sox3$^+$ (R,T) cells and reduced the number of Ngn2^{+HIGH} cells (P,T) in the Olig2$^+$ pMN domain (*n*=3). By contrast, Jag1 transfection did not affect the number of Hes5$^+$ (O), Ngn2^{+HIGH} (Q,T) or Sox3$^+$ (S,T) cells in the pMN Olig2$^+$ domain (*n*=3).

Fng augments Notch signalling within Dll1$^+$ progenitor domains, but obstructs Notch signalling in Jag1$^+$ p1 progenitors resulting in a reduced generation of MNs and an overproduction of V1 neurons.

Overexpression of Dll1 resulted in a significant reduction (~60%) of Isl1/Isl2$^+$ MNs 45-48 hours after electroporation (Fig. 4E,F) (le Roux et al., 2003). The expression of the pan-neuronal marker HuC/D was reduced to a similar extent in the MN domain (Fig. 4E,G), indicating that the loss of Isl1/Isl2 expression indeed reflected an enhanced suppression of neurogenesis by Dll1. Forced expression of Dll1 also suppressed the generation of Evx1/Evx2$^+$ V0 neurons (by ~60%; data not shown), but had a marginal effect on the production of En1$^+$ V1 neurons (~11%; Fig. 4E,H,I). In corresponding experiments, ectopic expression of Jag1 had a limited influence on the production of MNs and V0 neurons (13% and 18%, respectively), but suppressed the generation of V1 neurons by ~60% (Fig. 4E,J-M; data not shown). These effects of Dll1 and Jag1 could not be explained by a regional effect on the rate of apoptosis (see Fig. S4M-O in the supplementary material). Thus, forced Dll1 expression suppresses MNs and V0 neurons derived from Dll1$^+$/Fng$^+$ progenitors but has little effect on V1 neurons generated from Jag1$^+$/Fng$^-$ progenitors. Conversely, Jag1 effectively suppresses neurogenesis in Jag1$^+$/Fng$^-$ progenitor domains but has a limited influence on neurons generated from Dll1$^+$/Fng$^+$ progenitors. Together, these findings indicate that the patterned distribution of Fng proteins modulates the capacity of progenitor cells to respond to Dll1 and Jag1 signalling. Nevertheless, in domains in which Dll1 or Jag1 mediate the suppression of neurogenesis, their effect appears to be mediated through canonical Notch downstream signalling. For instance, the suppression of MN differentiation by overexpression Dll1 in the pMN domain was associated with a selective increase in the number of Hes5$^+$ (Fig. 4N) and Sox3$^+$ progenitors (Fig. 4R,T), whereas the fraction of cells expressing high levels of Ngn2 was reduced (Fig. 4P,T). By contrast, there was no significant change in Ngn2 (Fig. 4Q,T) expression or the numbers of Hes5$^+$ and Sox3$^+$ cells (Fig. 4O,S,T) within the pMN domain in similar experiments with Jag1.

DISCUSSION

HD proteins, which are implicated in pattern formation, as well as several components of the Notch pathway, exhibit specific expression domains along the DV axis of the neural tube, but their internal relationship has not been determined. We show that the patterned expression of Notch ligands and Fng genes are controlled by HD transcription factors. Loss of Nkx6.1 led to a ventral expansion of Jag1 expression, accompanied by a reduction of *Dll1*, *Lfng* and *Mfng* expression. Conversely, forced ectopic expression of Nkx6.1 suppressed the expression of Jag1 and induced that of *Dll1*, *Lfng* and *Mfng*. Perturbation of Dbx1 caused ectopic *Jag1* expression in the 'p0 domain' and a concomitant reduction of *Dll1*, *Lfng* and *Mfng* expression, whereas overexpression of Dbx1 had the opposite effect. By contrast, perturbation of Dll1 and Jag1 did not alter the expression patterns of the Nkx6.1 and Dbx1 proteins, and there was no obvious increase of cell intermingling at progenitor domain boundaries. In conclusion, these findings suggest a mechanism in which HD proteins act upstream of Notch ligands and Fng gene expression, resulting in the establishment of discrete progenitor domains with co-localized expression of Dll1 and Fng, whereas regions expressing Jag1 are devoid of Fng protein expression (see Fig. 5A).

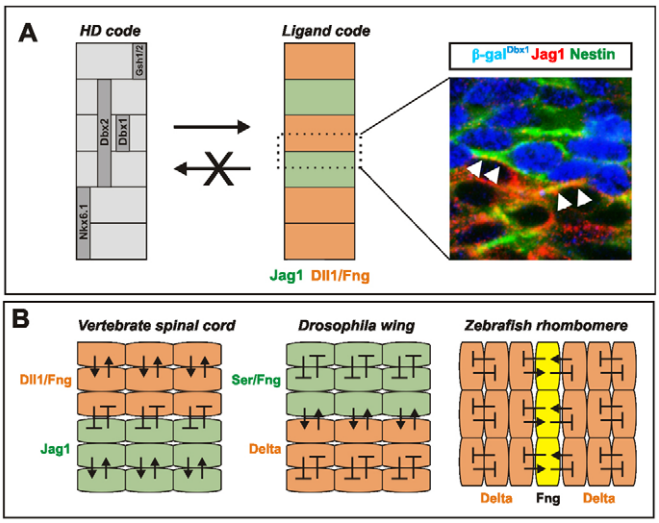

Fig. 5. Proposed model for the regulatory interactions between HD proteins and Notch pathway components, and downstream consequences for Notch signalling. (**A**) The spinal cord is regionalized into progenitor domains by the combinatorial expression pattern of HD proteins. This HD code specifies the different neuronal subtypes along the DV axis and delimits the expression of Notch ligands and Fng proteins to specific progenitor domains. Magnification of the boxed area shows the border between the p1 and p0 domains in *Dbx1*$^{\beta\text{-gal}/+}$ embryos at E10.5. Co-labelling with the cytoplasmic marker Nestin, membrane-bound Jag1 and nuclear β-gal demonstrates direct contact between p1 cells expressing Jag1 and Dbx1^{+} p0 cells. (**B**) The expression pattern of Fng relative to that of the Notch ligands directs Notch signalling to distinct positions within the vertebrate spinal cord, *Drosophila* wing and zebrafish hindbrain.

Our data reveal that genetic ablation of Notch ligand expression domains has several consequences for neuronal differentiation. Loss of Jag1 leads to an increase of V1-interneurons, but not of V0- or V2-interneurons or MNs. Loss of Dll1 has the opposite effect and results in an increase in the number of V0- and V2-interneurons and MNs, while leaving the number of V1-interneurons unaltered. Forced expression produced the converse picture in which Jag1 suppressed V1-interneuron differentiation, whereas Dll1 reduced the number of MNs. Interestingly, forced expression of Mfng had a similar activity to overexpression of Dll1 but opposite to that of Jag1 misexpression, which suggests that Mfng enhances Dll1/Notch signalling and reduces the efficiency of Jag1/Notch signalling. This is consistent with several previous observations (Stanley, 2007; Xu et al., 2007), but is in contrast to somitogenesis, in which Lfng appears to inhibit Dll-Notch signalling (Dale et al., 2003), and to data suggesting that Notch2 can respond to Jag1 in the presence of Fng (Hicks et al., 2000; Kopan and Ilagan, 2009).

Spatially organized expression of Notch ligands and Fng proteins is not unique to the DV axis of the developing spinal cord, but is found in many developmental processes, such as rhombomere organization along the anteroposterior axis of the developing hindbrain and DV patterning of the *Drosophila* imaginal wing and eye discs (Cheng et al., 2004; Wu and Rao, 1999). In sharp contrast to the developing spinal cord, however, Notch signalling activity in zebrafish rhombomeres and in the *Drosophila* wing and eye is constrained to boundaries between segments, rather than to an entire regional territory (Fig. 5B). More specifically, Notch activation regulates the segregation and differentiation of rhombomere boundary cells in the hindbrain (Cheng et al., 2004), whereas in the fly wing Notch activity is confined to a small group of cells at the wing margin that is important for wing outgrowth (Wu and Rao, 1999). We propose that this difference in outcome of Notch signalling (i.e. within a domain versus at or across the domain boundary) reflects the principal difference of how Notch ligand expression is set up relative to the pattern of Fng expression. In the *Drosophila* wing, the expression of Fng overlaps with that of Jag1 (Serrate in flies) but not with that of Dll1 (Delta in flies) (Wu and Rao, 1999), whereas in the developing spinal cord Fng expression is instead superimposed on Dll1-expressing, but not on Jag1-expressing, progenitor domains. As depicted in Fig. 5, the selection of which Notch ligand that Fng is co-expressed with has important consequences for signalling. In the *Drosophila* wing, the presence of Fng in the Serrate expressing domain precludes signalling within the Serrate^{+}/Fng^{+} territory, and the absence of Fng expression in the Delta-expressing domain precludes Notch signalling in the Delta^{+}/Fng^{-} domain. This internal relationship of Fng and Notch ligands, however, enables functional Notch signalling to take place at the domain boundary, as Delta ligands can activate Notch receptors in the neighbouring cells of the Serrate^{+}/Fng^{+} domain, and conversely Serrate ligands can activate Notch receptors in the neighbouring cells of the Delta^{+}/Fng^{-} domain (Fig. 5B). The situation in the developing spinal cord, with its Jag1^{+}/Fng^{-} and Dll1^{+}/Fng^{+} domains, generates the opposite outcome; a domain-wide activation by either Dll or Jag1, and a suppression of signalling across progenitor domain boundaries (Fig. 5B). This is in line with the observed phenotypes in gain- and loss-of-function experiments with Dll1, Jag1 and Mfng, in which the establishment of progenitor domain boundaries was not altered, but which revealed a domain-specific regulation of neurogenesis along the DV axis.

The establishment of the CNS depends on the generation of functionally distinct neurons produced in appropriate numbers and in a spatially defined manner. Previous findings demonstrate that the use of different Notch ligands can mediate qualitatively distinct responses in cell fate determination. For instance, Dll4 and Jag1 have opposing effects on angiogenesis (Benedito et al., 2009), and Dll4, but not Dll1, can function to specify V2a and V2b interneuron subtype identities in a uniform population of ventral progenitors of the developing spinal cord (Del Barrio et al., 2007; Peng et al., 2007; Rocha et al., 2009) (see also Fig. 3). Despite the fact that Dll1 and Jag1 control the differentiation of distinct neuronal subtypes along the DV axis, our data do not support the notion that signalling downstream of Dll1 and Jag1 is qualitatively different, or that Dll1 or Jag1 would determine the actual identity of cells. Instead, at least with regard to the regulation of Hes5, Ngn2 and Sox3 expression, Dll1 and Jag1 appear to carry out similar signalling activities, albeit in distinct DV progenitor domains. The subtype identity of cells is therefore more likely to be determined through the regional activity of cell fate-determining HD proteins (Jessell, 2000), while the specific distribution of Notch ligands and Fng proteins along the DV axis, also controlled by HD proteins, endows distinct progenitor domains with an enhanced regulatory autonomy and the possibility to influence the pace of neurogenesis in a domain- and cell type-specific manner along the DV axis of the neural tube (Fig. 5). Hence, the combined functional activities and regulatory interactions between HD proteins and components of Notch signalling described here should contribute to a coordinated control of cell-type specification and regulation of cell number in the developing CNS.

Acknowledgements
We thank A. Pierani for providing the Dbx1 mutant mice, M. Sander for the Nkx6.1 mice and G. Sturm for the Jagged$^{Ndr/+}$ mice. We are also grateful to D. Anderson, A. Pierani, M. Goulding, T. Jessell, B. Novitch, T. Edlund and C. Tabin for supplying us with reagents. We are grateful to A Kjaeldgaard for providing us with abortion material. This work was supported by the Swedish Research Council (DBRM), the Swedish Foundation for Strategic Research (FFL-2, CEDB and OBOE), EU project EuroSyStem, the Göran Gustafsson Foundation, Swedish Brain Power, the Swedish Cancer Society, the Craig Hospital Foundation and the research funds of the Karolinska Institutet.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.036806/-/DC1

References
Akazawa, C., Sasai, Y., Nakanishi, S. and Kageyama, R. (1992). Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. *J. Biol. Chem.* **267**, 21879-21885.
Andersson, E., Jensen, J. B., Parmar, M., Guillemot, F. and Bjorklund, A. (2006). Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2. *Development* **133**, 507-516.
Baek, J. H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T. and Kageyama, R. (2006). Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. *Development* **133**, 2467-2476.
Benedito, R., Roca, C., Sorensen, I., Adams, S., Gossler, A., Fruttiger, M. and Adams, R. H. (2009). The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* **137**, 1124-1135.
Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.
Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-445.
Castro, D. S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I. J., Parras, C., Hunt, C., Critchley, J. A., Nguyen, L., Gossler, A., Gottgens, B. et al. (2006). Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. *Dev. Cell* **11**, 831-844.
Cheng, Y. C., Amoyel, M., Qiu, X., Jiang, Y. J., Xu, Q. and Wilkinson, D. G. (2004). Notch activation regulates the segregation and differentiation of rhombomere boundary cells in the zebrafish hindbrain. *Dev. Cell* **6**, 539-550.
Dale, J. K., Maroto, M., Dequeant, M. L., Malapert, P., McGrew, M. and Pourquie, O. (2003). Periodic notch inhibition by lunatic fringe underlies the chick segmentation clock. *Nature* **421**, 275-278.
Del Barrio, M. G., Taveira-Marques, R., Muroyama, Y., Yuk, D. I., Li, S., Wines-Samuelson, M., Shen, J., Smith, H. K., Xiang, M., Rowitch, D. et al. (2007). A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool. *Development* **134**, 3427-3436.
Dubreuil, V., Hirsch, M. R., Jouve, C., Brunet, J. F. and Goridis, C. (2002). The role of Phox2b in synchronizing pan-neuronal and type-specific aspects of neurogenesis. *Development* **129**, 5241-5253.
Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V. and Jessell, T. M. (1997). Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 451-466.
Helms, A. W. and Johnson, J. E. (2003). Specification of dorsal spinal cord interneurons. *Curr. Opin. Neurobiol.* **13**, 42-49.
Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-790.
Herranz, H. and Milan, M. (2006). Notch and affinity boundaries in Drosophila. *BioEssays* **28**, 113-116.
Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F. and Weinmaster, G. (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat. Cell Biol.* **2**, 515-520.
Hrabe de Angelis, M., McIntyre, J., 2nd and Gossler, A. (1997). Maintenance of somite borders in mice requires the Delta homologue Dll1. *Nature* **386**, 717-721.
Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.
Johnston, S. H., Rauskolb, C., Wilson, R., Prabhakaran, B., Irvine, K. D. and Vogt, T. F. (1997). A family of mammalian Fringe genes implicated in boundary determination and the Notch pathway. *Development* **124**, 2245-2254.
Kageyama, R. and Nakanishi, S. (1997). Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. *Curr. Opin. Genet. Dev.* **7**, 659-665.
Kopan, R. and Ilagan, M. X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* **137**, 216-233.
le Roux, I., Lewis, J. and Ish-Horowicz, D. (2003). Notch activity is required to maintain floorplate identity and to control neurogenesis in the chick hindbrain and spinal cord. *Int. J. Dev. Biol.* **47**, 263-272.
Lee, S. K., Lee, B., Ruiz, E. C. and Pfaff, S. L. (2005). Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. *Genes Dev.* **19**, 282-294.
Lindsell, C. E., Boulter, J., diSibio, G., Gossler, A. and Weinmaster, G. (1996). Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. *Mol. Cell. Neurosci.* **8**, 14-27.
Louvi, A. and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat. Rev. Neurosci.* **7**, 93-102.
Matise, M. P. and Joyner, A. L. (1997). Expression patterns of developmental control genes in normal and Engrailed-1 mutant mouse spinal cord reveal early diversity in developing interneurons. *J. Neurosci.* **17**, 7805-7816.
Mizuguchi, R., Sugimori, M., Takebayashi, H., Kosako, H., Nagao, M., Yoshida, S., Nabeshima, Y., Shimamura, K. and Nakafuku, M. (2001). Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. *Neuron* **31**, 757-771.
Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. et al. (2000). Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-375.
Muhr, J., Andersson, E., Persson, M., Jessell, T. M. and Ericson, J. (2001). Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. *Cell* **104**, 861-873.
Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. *Dev. Biol.* **174**, 233-247.
Novitch, B. G., Chen, A. I. and Jessell, T. M. (2001). Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. *Neuron* **31**, 773-789.
Peng, C. Y., Yajima, H., Burns, C. E., Zon, L. I., Sisodia, S. S., Pfaff, S. L. and Sharma, K. (2007). Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron* **53**, 813-827.
Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.
Pierani, A., Moran-Rivard, L., Sunshine, M. J., Littman, D. R., Goulding, M. and Jessell, T. M. (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. *Neuron* **29**, 367-384.
Poh, A., Karunaratne, A., Kolle, G., Huang, N., Smith, E., Starkey, J., Wen, D., Wilson, I., Yamada, T. and Hargrave, M. (2002). Patterning of the vertebrate ventral spinal cord. *Int. J. Dev. Biol.* **46**, 597-608.
Przemeck, G. K., Heinzmann, U., Beckers, J. and Hrabe de Angelis, M. (2003). Node and midline defects are associated with left-right development in Delta1 mutant embryos. *Development* **130**, 3-13.
Rocha, S. F., Lopes, S. S., Gossler, A. and Henrique, D. (2009). Dll1 and Dll4 function sequentially in the retina and pV2 domain of the spinal cord to regulate neurogenesis and create cell diversity. *Dev. Biol.* **328**, 54-65.
Rowitch, D. H. (2004). Glial specification in the vertebrate neural tube. *Nat. Rev. Neurosci.* **5**, 409-419.
Sakamoto, K., Yan, L., Imai, H., Takagi, M., Nabeshima, Y., Takeda, S. and Katsube, K. (1997). Identification of a chick homologue of Fringe and C-Fringe 1, involvement in the neurogenesis and the somitogenesis. *Biochem. Biophys. Res. Commun.* **234**, 754-759.
Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T. M. and Rubenstein, J. L. (2000). Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. *Genes Dev.* **14**, 2134-2139.
Scardigli, R., Schuurmans, C., Gradwohl, G. and Guillemot, F. (2001). Crossregulation between Neurogenin2 and pathways specifying neuronal identity in the spinal cord. *Neuron* **31**, 203-217.
Scardigli, R., Baumer, N., Gruss, P., Guillemot, F. and Le Roux, I. (2003). Direct and concentration-dependent regulation of the proneural gene Neurogenin2 by Pax6. *Development* **130**, 3269-3281.
Shimizu, K., Chiba, S., Saito, T., Kumano, K., Takahashi, T. and Hirai, H. (2001). Manic fringe and lunatic fringe modify different sites of the Notch2 extracellular region, resulting in different signaling modulation. *J. Biol. Chem.* **276**, 25753-25758.
Stanley, P. (2007). Regulation of Notch signaling by glycosylation. *Curr. Opin. Struct. Biol.* **17**, 530-535.
Sugimori, M., Nagao, M., Bertrand, N., Parras, C. M., Guillemot, F. and Nakafuku, M. (2007). Combinatorial actions of patterning and HLH

transcription factors in the spatiotemporal control of neurogenesis and gliogenesis in the developing spinal cord. *Development* **134**, 1617-1629.
Taylor, M. K., Yeager, K. and Morrison, S. J. (2007). Physiological Notch signaling promotes gliogenesis in the developing peripheral and central nervous systems. *Development* **134**, 2435-2447.
Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.
Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M. and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. *Neuron* **31**, 743-755.
Wu, J. Y. and Rao, Y. (1999). Fringe: defining borders by regulating the notch pathway. *Curr. Opin. Neurobiol.* **9**, 537-543.
Xu, A., Haines, N., Dlugosz, M., Rana, N. A., Takeuchi, H., Haltiwanger, R. S. and Irvine, K. D. (2007). In vitro reconstitution of the modulation of Drosophila Notch-ligand binding by Fringe. *J. Biol. Chem.* **282**, 35153-35162.