A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool

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In the developing central nervous system, cellular diversity depends in part on organising signals that establish regionally restricted progenitor domains, each of which produces distinct types of differentiated neurons. However, the mechanisms of neuronal subtype specification within each progenitor domain remain poorly understood. The p2 progenitor domain in the ventral spinal cord gives rise to two interneuron (IN) subtypes, V2a and V2b, which integrate into local neuronal networks that control motor activity and locomotion. Foxn4, a forkhead transcription factor, is expressed in the common progenitors of V2a and V2b INs and is required directly for V2b but not for V2a development. We show here in experiments conducted using mouse and chick that Foxn4 induces expression of delta-like 4 (Dll4) and Mash1 (Ascl1). Dll4 then signals through Notch1 to subdivide the p2 progenitor pool. Foxn4, Mash1 and activated Notch1 trigger the genetic cascade leading to V2b INs, whereas the complementary set of progenitors, without active Notch1, generates V2a INs. Thus, Foxn4 plays a dual role in V2 IN development: (1) by initiating Notch-Delta signalling, it introduces the asymmetry required for development of V2a and V2b INs from their common progenitors; (2) it simultaneously activates the V2b genetic programme.

KEY WORDS: Notch1, Delta-like 4, Foxn4, Gata, Scl (Tal1), Chx10, Spinal cord, Neurogenesis, Chick, Mouse, V2 interneurons, Mash1 (Ascl1)

INTRODUCTION

The neurons and glial cells of the mature central nervous system (CNS) develop from the neuroepithelial progenitor cells that surround the lumen of the embryonic spinal cord and the ventricles of the brain – the so-called ventricular zone (VZ). The spinal cord VZ is a mosaic of progenitor cell domains, each of which generates one or more distinct subtypes of neurons followed by glial cells. The domain pattern is established in response to signals from local organising centres (Briscoe et al., 2000; Ericson et al., 1997). For example, sonic hedgehog diffusing from the notochord and floor plate forms a concentration gradient that specifies five ventral progenitor domains known as p3, pMN, p2, p1 and p0 (ventral to dorsal). This initial patterning phase is followed by the neurogenic phase, during which the progenitor domains give rise to particular combinations of differentiated neurons and glia (Rowitch, 2004). Motor neurons and several types of interneurons (INs) in the ventral spinal cord assemble into local networks that generate the rhythmic output required for locomotion (reviewed by Kiehn, 2006). To understand how locomotor circuits develop, it is necessary to understand the genetic and cellular mechanisms that determine neuron diversity.

The p2 progenitor domain generates two distinct subtypes of INs, V2a and V2b (Karunaratne et al., 2002; Li et al., 2005; Smith et al., 2002; Zhou et al., 2000). Postmitotic V2a INs are characterised by expression of the homeodomain transcription factor Chx10 (Ericson et al., 1997), whereas V2b INs express transcription factors Gata2, Gata3 and Scl (Tal1) (Karunaratne et al., 2002; Muroyama et al., 2005; Smith et al., 2002). How V2 INs incorporate into the local spinal circuitry is not established, although V2a INs are thought to be excitatory (glutamatergic) and to project ipsilaterally (Kiehn, 2006; Kimura et al., 2006). The neurotransmitter phenotype of V2b INs is not known. V2a and V2b INs are derived from common progenitors that initially express the forkhead/winged helix transcription factor Foxn4 (Li et al., 2005) (this paper). How does this homogeneous progenitor pool generate two distinct neuronal subtypes?

The Notch-Delta signalling pathway is often used to establish or to maintain differences between lineally related cells (Artavanis-Tsakonas et al., 1999; Louvi and Artavanis-Tsakonas, 2006). For example, signalling between Notch1 and its ligand delta-like 4 (Dll4) in endothelial cells is necessary for artery-vein discrimination and also for sprouting of lymphatic vessels from veins (Duarte et al., 2004; Seo et al., 2006). We thought it possible that the distinction between V2a and V2b INs might also be established through Notch-Delta signalling. Notch1, 2 and 3 are all expressed directly in the ventral VZ of the embryonic spinal cord (Lindsell et al., 1996), as are their ligands Dll1, Dll3, Dll4 and jagged 1 (Braun et al., 2000; Lindsell et al., 1996; Mailhos et al., 2001). Unlike Dll1 and Dll3, which are expressed widely throughout the VZ and/or in postmitotic neurons, Dll4 appears to be restricted to the p2 domain of the VZ, suggesting a specific role in V2 IN development (Benedito and Duarte, 2005).
We have examined the relationship between Foxn4 and Notch-Delta signalling during development of V2a and V2b sub-lineages. We demonstrated that Foxn4 is a master regulator of the V2b sub-lineage. Delta signalling during development of V2a and V2b sub-lineages. We also found that Foxn4 controls determinants Gata2, Gata3 and Scl, while repressing markers of lineage, being necessary and sufficient to induce the V2b sub-lineage. Notch1 was conditionally inactivated, V2a INs were overproduced which then generate V2b INs under the combined action of Foxn4, Notch1 and Mash1; (3) the complementary set of progenitors fails to activate Notch1 and consequently generates V2a INs.

**Materials and Methods**

**Transgenic mice**

We used tissue from the following mutant mice: Foxn4<sup>−/−</sup> (Li et al., 2004), Scl conditional nulls (ΔScl) (Muroyama et al., 2005), Notch1 conditional nulls (Yang et al., 2006), Mash1<sup>−/−</sup> (Guillemot et al., 1993). The Scl and Notch1 conditional nulls were crossed to Nestin-Cre to eliminate the floxed alleles throughout the CNS.

**Electroporation constructs**

The complete coding sequence of mouse Foxn4 was cloned from an E15.5 mouse eye cDNA library by PCR with the primers 5′-CTCCAGGAAAATGATAGAAGATGTG and 5′-CTGCCAGAAGATGGTAGGTAGGAG. The cloned sequence matched the published mouse Foxn4 mRNA (GenBank accession AY039039), with the exception of nucleotide T288G, which does not change the translated protein sequence. The cDNA (from ATG to stop codon) was cloned into the pCA<sub>IRES-eGFP</sub>-LINK-IRES<sub>CAGGS</sub>-F<sub>ClaI</fsub> bi-cistronic expression vector (Schubert and Lumsden, 2005) by PCR.

The Mash1 vector (gift from Francois Guillemot, National Institute for Medical Research, London, UK) contains the coding sequence of mouse Mash1 under transcriptional control of a synthetic β-actin promoter (CAGGS), followed by IRES-eGFP (with a nuclear localisation signal).

A human DLL4 (hDLL4) expression vector was kindly provided by Ji-Liang Li (John Radcliffe Hospital, University of Oxford, UK). The full-length human DLL4 coding sequence was PCR-amplified from human placental cDNA, using primers 5′-GGATCCCATATGGCGGC AGC -GTCCCGTAGCGCCT and 5′-GGATCCCATATGGCGGC AGC -GGATCCCATATGGCGGC AGC. The hDLL4 was released from the pGEM-T Easy vector (Promega) by BamHI digestion and inserted into pCDNA3.1/myc-His (Invitrogen).

**Electroporation of chick embryos in ovo**

Fertilised chicken eggs were incubated at 38°C in a humidified incubator, opened and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos were electroporated at st11-16 (Itasaki et al., 1999). The expression constructs [2-5 μg/μl in PBS and 0.8% (w/v) Fast Green] were injected into the lumen of the spinal cord and electroporated using an Intracel TSS20 Ovodyne electroporator with EP21 current amplifier and 0.5 mm diameter home-made platinum electrodes (4-5 pulses of 20-25 volts for 50 milliseconds each).

**Tissue preparation and immunohistochemistry**

Embryos were dissected in cold PBS and fixed in 4% (w/v) paraformaldehyde in PBS. They were then cryo-protected with 20% (w/v) sucrose in PBS, embedded in OCT and frozen for cryo-sectioning (10 μm nominal thickness). The antibodies used were: rabbit polyclonal anti-GFP at 1:8000 (ab290-50, Abcam), rabbit anti-Chx10 at 1:100 (provided by...
Thomas Jessell, Columbia University, NY and Connie Cepko, Harvard Medical School, Boston, MA), mouse monoclonal anti-Myc at 1:200 (M4439, Sigma), mouse monoclonal anti-Gata3 at 1:100 (SC268, Santa Cruz), rabbit anti-Olig2 1:8000 (provided by Charles Stiles, Dana Farber Cancer Institute, Boston, MA), mouse monoclonal anti-Hb9 (Developmental Studies Hybridoma Bank, DSHB), rabbit anti-β-gal at 1:2000 (Cappel, ICN Pharmaceuticals), mouse anti-Lim1/2 (Lhx1/5) at 1:30 (DSHB), mouse anti-En1 at 1:5 (DSHB), mouse anti-β-gal (Promega) at 1:300 (with tyramide amplification, Molecular Probes). Some of the sections were incubated with DAPI in PBS in order to visualise cell nuclei before mounting.

In situ hybridisation (ISH)
Our ISH protocols are as described (http://www.ucl.ac.uk/~ucbzwdr/richardson.htm). Some of the templates used to make in situ hybridisation probes were cDNAs obtained by RT-PCR (Invitrogen Kit 11904-018) from a ventral spinal cord chicken cRNA library (Ivanova et al., 2004). PCR forward and reverse primers were as follows: Foxn4, 5'-CCCCATG-GCTGGAAAAACTC and 5'-AGAGGTGAGGAGGAGGTGTG; Lhx3, 5'-AGAGCGAGCTGGCCGAGGAAGTG and 5'-TGTCCTCATGATGCC-CAAACC; Chx10, 5'-AACATCTCCTAATCCCAACTG and 5'-GTGCATATCTCAAACACCTCAAT; Gata2, 5'-GAGCGGCTCACTC-TATTACC and 5'-TTGCGACACCTACATTCTCCT; Gata3, 5'-AAAGCTCTTGCCCCACCGACTC and 5'-GGACATCAGACCCATAACCCACG.

A longer chick Foxn4 template was cloned by RNA ligation-mediated rapid amplification of 5’ and 3’ cDNA ends (RLM-RACE) (Gene Racer Kit), using the supplied 5’ upper primer and 5’-GCCAGAGCTTGAGGAGGTGTG. The cDNA product was 850 bp. The template for chicken Dll4 was plasmid ChEST714c11 (ARK-Genomics) cut with Dll4 of the mouse

RESULTS
Foxn4 is a master regulator of V2b INs
Foxn4 expression has been described in the developing mouse retina and neural tube (Gouge et al., 2001; Li et al., 2004; Li et al., 2005). In the ventral neural tube it is expressed specifically in the p2 progenitor domain (Li et al., 2005), which generates V2a and V2b INs. We analysed Foxn4 expression in chick embryos by in situ hybridisation (ISH) during Hamburger-Hamilton stages 10 to 25 (st10-25). We first detected small numbers of Foxn4-positive cells in the rostral spinal cord at st13 (see Fig. S1A in the supplementary material). At later stages, the number of Foxn4-positive cells increased. As in the mouse, a few cells were present in the VZ close to the lumen, but most accumulated towards the outer margin of the VZ (see Fig. S1B,C in the supplementary material). They are generated exclusively in the p2 progenitor domain, within the region of Nkx6.1 expression but immediately dorsal to the Olig2-expressing pMN domain (see Fig. S1D in the supplementary material and data not shown).

We compared the expression of Foxn4 with Chx10, which marks V2a INs (Ericson et al., 1997), and with Gata2, which marks V2b INs (Karunaratne et al., 2002), in chick embryos. There was a significant degree of overlap between Foxn4 and Gata2 (see Fig. S1E,G in the supplementary material) but no overlap between Foxn4 and Chx10 (see Fig. S1F,H in the supplementary material), implicating Foxn4 in the development of V2b but not V2a INs. In support of this, we found that electroporation of β-actin-Foxn4-ires-GFP into st12-14 chick spinal cord could induce ectopic expression of the V2b actin-Foxn4, Gata2 and Scl, but was unable to induce ectopic V2a markers Chx10 (0/15 embryos) or Lhx3 (0/7 embryos) (Fig. 1A-F,1J). Gata2 was induced robustly by 24 hours post-electroporation (50/50 embryos), whereas Scl and Gata3 required longer (0/5 embryos after 24 hours versus 17/17 embryos after 48 hours for Scl; 0/5 embryos after 24 hours versus 6/8 embryos after 48 hours for Gata3). The number of Chx10-positive V2a INs generated from the p2 progenitor domain was reduced markedly in these experiments (62±9% reduction, mean±s.e.; 107 cells on the control side versus 33 on the electroporated side; 41 sections from eight embryos) (Fig. 1G,H).

This suggested that Foxn4 might act as a master regulator of the V2b sub-lineage. In a further test of this idea we asked whether Foxn4 can repress alternative IN fates in more-dorsal progenitor domains. We found that electroporated Foxn4 inhibited expression of engrailed 1 (En1), a marker of postmitotic V1 INs (Ericson et al., 1997), and of Lhx1/5, which marks postmitotic INs derived from dorsal progenitor domains dP1-dP6 with the
exception of dP3 (reviewed by Lewis, 2006). A reduction of 31±3% (mean±s.e., n=4) was observed for En1 (1173 cells on the control side versus 776 on the electroporated side; 39 sections from four embryos; see Fig. S2A in the supplementary material) and a reduction of 42±13% (n=4) for Lhx1/5 (4239 cells on the control side versus 2604 on the electroporated side; 30 sections from four embryos; see Fig. S2B in the supplementary material). These experiments suggest that ectopic expression of Foxn4 can reprogram progenitors to a V2b IN fate.

It has been reported that Scl function is necessary and sufficient for V2b IN development and is required for the maintenance of normal levels of Gata2 expression (Muroyama et al., 2005). We therefore explored the genetic relationship between Scl and Foxn4. There was a small but significant overlap between Foxn4 and Scl in wild-type mice (Fig. 2A). Scl mRNA expression was abolished in Foxn4 mutant mouse spinal cords at E10.5 (3/3 embryos) and E11.5 (2/2 embryos) (Fig. 2D,E and data not shown). Conversely, Foxn4 was expressed as normal in Scl conditional null mice (2/2 embryos) (Fig. 2F,G). Also, as described above, Foxn4 induces Scl expression after 48 hours (17/17 embryos) (Fig. 1D, Fig. 2C). Therefore, it seems that Foxn4 lies upstream of Scl in the genetic hierarchy leading to V2b INs.

A negative-control vector with inverted Foxn4 sequences has been used in parallel with all experiments reported above, without any activity (data not shown). Taken together, our data suggest that Foxn4 is a master regulator of the V2b sub-lineage. Furthermore, we have shown that Scl lies downstream of Foxn4 in the pathway that governs development of V2b INs.

Foxn4 is expressed in the common progenitors of V2a and V2b INs

It was previously reported that V2a and V2b INs share common, Foxn4-expressing progenitor cells in the VZ (Li et al., 2005). We confirmed this by following expression of β-galactosidase (β-gal) in mouse Foxn4+/- heterozygotes, which is possible because the knockout allele contains a functional copy of lacZ under Foxn4 transcriptional control. By double immunohistochemistry we found that β-gal protein was present in cells that co-express Chx10 (Fig. 3A), as well as in cells that express Gata3 (Fig. 3B). By contrast, Foxn4 transcripts or protein were never found in the same cells as Chx10 or Gata3 (see Fig. S1H in the supplementary material) (Li et al., 2005). The most parsimonious interpretation is that there is a common pool of Foxn4-positive progenitors that generates both V2a and V2b INs. The reason that β-gal can be detected in differentiated V2a as well as V2b INs is presumably because it has a longer half-life than Foxn4. In further support of the existence of a common pool of V2a/V2b progenitors, we found that those Foxn4-positive cells that lie closest to the lumen (where neural progenitors undergo mitosis) co-express the V2a determinant Lhx3 (Fig. 3D), as well as Gata2 (see Fig. 1G in the supplementary material) and Mash1 (Fig. 3C, Fig. 6A).

Foxn4 activates delta-like 4 in p2 progenitors

mRNA encoding the Notch ligand delta-like-4 (Dll4) is expressed in scattered cells in mouse and chicken within the p2 progenitor domain (Fig. 4A,B and data not shown). Some of the Dll4-positive cells in the p2 domain co-expressed Foxn4 (Fig. 4A,B). Many of these Foxn4/Dll4 double-positive cells were found at the ventricular surface, where mitosis occurs. Double-positive cells frequently occurred as cell pairs (arrows in Fig. 4B, shown at higher magnification in C,D). These images strongly suggest that Dll4 and Foxn4 are co-expressed in cells that are dividing, or in recently separated siblings that are still in contact.

To determine whether Dll4 and Foxn4 interact genetically, we performed chick electroporation experiments at st11-12 with β-actin-Foxn4-ires-gfp. Foxn4 induced ectopic expression of Dll4 at 34 hours post-electroporation in 12/12 embryos analysed (Fig. 4F). A control vector with inverted Foxn4 sequences had no such effect (data not shown). Consistent with these observations, Dll4 expression was abolished in the p2 domain of Foxn4-null mice at E10.5 (3/3 embryos analysed) and E11.5 (2/2 embryos analysed) (Fig. 4E and data not shown). We conclude that Foxn4 is necessary and sufficient for activation of Dll4 in p2 progenitors.

Dll4 inhibits V2a lineage progression

To discover whether Dll4 is involved in the specification of V2 INs – possibly through its interactions with Notch – we performed gain-of-function experiments in chick neural tube by electroporating an expression vector encoding human DLL4 (CMV-hDll4-Myc). We performed two sets of experiments. In the first, we electroporated at st11-13 and analysed the embryos after a further 44 hours (st19-20). In 16 embryos analysed, we found no ectopic induction of Chx10 immunoreactivity or Gata2 mRNA. By contrast, a reduction of Chx10 and Gata2 expression was observed on the electroporated versus the control side, Chx10 being more strongly repressed (~80% reduction) than Gata2 (~50% reduction) (63 Chx10-positive cells on the control side versus 12 on the electroporated side, compared with 90 Gata2-positive cells on the control side versus 58 on the electroporated side; 24 sections from four embryos; data not shown).

In the second set of experiments, we electroporated at st14-16 and analysed the embryos after a further 48 hours (st21-23). In this set of experiments, 15 embryos were analysed for Chx10 immunoreactivity and Chx10, Scl and Gata2 mRNA (Fig. 5). As in the first experiment,
Mash1 induces Mash1 in the p2 domain
The extensive overlap of Mash1 and Foxn4 expression in the mouse p2 domain (Fig. 3C, Fig. 6A) suggested some form of regulatory relationship. We therefore explored the interactions between Foxn4 and Mash1 in more detail. We confirmed the finding of Li et al. (Li et al., 2005) that Foxn4 is expressed as normal in Mash1-null spinal cord (Fig. 6C,D). After electroporating β-actin-Foxn4-IRES-GFP in the chick spinal cord at st13-14, we found strong ectopic induction of Cash1 (the chick homologue of Mash1) after 24 hours (6/6 embryos) and 48 hours (3/5 embryos; Fig. 6B and data not shown). The negative control vector with inverted Foxn4 sequences had no activity (data not shown). These experiments indicate that Foxn4 is upstream of and controls expression of Mash1 in p2, and fits with the observation that Mash1 expression in p2 is lost in Foxn4-null mice (Li et al., 2005).

Mash1 stimulatesDll4 expression but does not induce V2b INs
Mash1 controls the expression of Dll4 in the ventral telencephalon and dorsal spinal cord (Casarosa et al., 1999), so we asked whether Mash1 can also induce Dll4. We electroporated full-length mouse Mash1 (β-actin-Mash1-IRES-GFP) into st13-14 chick neural tube. After 24 hours of incubation, 8/8 embryos showed clear ectopic induction of Dll4 on the electroporated side (Fig. 6E). After 48 hours, 5/5 embryos displayed weaker but still clear induction of Dll4 (data not shown). In none of the 13 embryos analysed did we find any ectopic expression of Chx10, Gata2 or Scl transcripts or Chx10 immunoreactivity (Fig. 6F,G,H,H* and data not shown). On the other hand, we observed a loss of endogenous Chx10-positive INs in the p2 domain of 5/5 embryos analysed (76±6% reduction, n=23) (Fig. 6F,G,H,G), with little or no concomitant reduction of Gata2 or Scl (Fig. 6H,H*). These data suggest that induction of Dll4 and consequent repression of Chx10-positive V2a INs by Foxn4 might be mediated indirectly via Mash1. However, we found that Dll4 is expressed as normal at E10.5-11 in Mash1-null embryos (4/4 embryos; Fig. 6L,J). Therefore, Mash1 might be involved in maintaining or reinforcing Dll4 expression but is not required for its initiation. Although Mash1 is necessary to develop the V2b fate (Li et al., 2005), it is not sufficient to do so, judging by its inability to induce ectopic Gata2 or Scl expression. Therefore, it appears that the V2b program of gene expression is absolutely dependent on Foxn4.

Notch1 is required for generation of V2b INs
The fact that Dll4 preferentially represses the V2a fate suggests that the Notch-Delta system might be responsible for the V2a-V2b binary fate decision in p2 progenitors. To test this, we analysed Notch1 mutant (cKO) mouse embryos at E10.5 and E11.5 by ISH for Foxn4 or Scl, or by immunohistochemistry for Chx10, Gata3, Olig2 or Hb9 (Hixb9) (Fig. 7). Olig2 is a basic helix-loop-helix transcription factor that is expressed in the progenitors of motor neurons (MNs) and oligodendrocytes but not in postmitotic MNs (Lu et al., 2000), whereas Hb9 is a transcription factor expressed in early committed oligodendrocytes.

there was no ectopic expression of Chx10 protein or mRNA but a strong repression of Chx10 protein on the electroporated versus control side (51±5% reduction, mean±s.e.; 137 sections from 13 embryos; two-tail t-test=3.6 at P=0.001) (Fig. 5A,B). Gata2 mRNA was expressed ectopically in some embryos (19/63 sections in five out of 15 embryos). In general, the induction of Gata2 was modest and always restricted to the p1-p0 domain (Fig. 5D). Signal (estimated by counting pixels with ImageJ) was not significantly different on the electroporated versus control sides (594±83 versus 562±83 pixels, respectively; 80 sections from 7 embryos; two-tail t-test=0.3 at P=0.8, not significant) (Fig. 5D’). The Scl signal was also not significantly different between electroporated and control sides (396±64 pixels versus 361±57, respectively; 86 sections from 9 embryos; two-tail t-test=0.5 at P=0.6, not significant), nor was there any ectopic expression of Scl (Fig. 5D’). These results suggest that at st14-16, Dll4 overexpression specifically represses the V2a fate with little or no effect on V2b fate. In Dll4 electroporations, some cells were Dll4-Myc/Chx10 double positive (Fig. 5C), indicating that expression of Dll4 is compatible with expression of Chx10 in the same cell.
MNs (Thaler et al., 1999). At E11.5, no Gata3 (0 versus 99±3, n=14 sections from three embryos; two-tail t-test=26, P<0.001) or Scl-positive cells were present in the ventral spinal cord of Notch1 cKO mice (3/3 embryos analysed) (Fig. 7A-D,M-N). Instead, twice the normal number of Chx10-positive cells was observed (200±10 versus 102±3, n=16 sections from three embryos; two-tail t-test=8.6 at P<0.001) (Fig. 7A-D), as previously reported (Yang et al., 2006). pMN progenitors that express Olig2 were drastically reduced at this age (2±0.5 versus 39±2, n=14 sections from three embryos; two-tail t-test=17, P<0.001), but the number of Hb9-positive cells was not significantly affected in the Notch1 mutant (3/3 embryos analysed) by comparison with wild-type mice (175±16 versus 165±8, n=14 sections from three embryos; two-tail t-test=0.6, P=0.6) (Fig. 7D,E-F). It seems that Notch1 signalling is necessary to prevent premature differentiation of most progenitor cells in the ventral cord, judging by the loss of the ventral VZ in the mutant (Yang et al., 2006). However, loss of Notch1 does not seem to result in respecification of pMN progenitors to p2 progenitors, as originally proposed (Yang et al., 2006). Rather, the phenotype is more consistent with respecification of V2b to V2a INs, consistent with the idea that signalling through Notch1 is required for V2b IN development.

Foxn4 is very much reduced in the E11.5 Notch1 conditional null spinal cord (Fig. 7I,J, arrow). A simple interpretation is that the Foxn4-positive progenitors of V2a and V2b INs differentiate prematurely and completely into V2a INs in the mutant and, in doing so.

![Fig. 5. Dll4 inhibits V2a lineage progression. Chick embryos were electroporated with human Dll4 (hDll4) in the form of hDll4-Myc at st14-16 and analysed after 48 hours. (A) Double immunolabelling for Chx10 (red) and hDll4-Myc (green) showing repression of Chx10-positive cells. (B) Quantification of Chx10-labelled cells showed a ~50% decrease on the hDll4-electroporated side compared with the contralateral, control side. (C) Some hDll4-electroporated cells co-express Chx10, consistent with the idea that Dll4 can suppress V2a generation in a non-cell-autonomous fashion. (D) Immunolabelling for hDll4-Myc (green). (D') Dll4 exceptionally can induce Gata2 (arrowhead). (D'') Dll4 does not affect Scl expression. (E) Dll4 does not greatly affect generation of V2b INs, judging by ISH. Quantification of V2b markers Gata2 and Scl by pixel-counting software showed no significant effect on V2b production (see text for statistics).](image)

![Fig. 6. Foxn4 controls Mash1 expression. (A) Double ISH for Foxn4 (red) and Mash1 (green) in E10.5 mouse cord. Note the extensive overlap in the p2 domain. (B,B') Foxn4 induces ectopic expression of Cash1 in chick electroporation experiments. (C,D) Foxn4 expression does not depend on Mash1; there is no noticeable change in the Foxn4 ISH signal in Mash1-null mice compared with wild type. (E,E') Electroporation of β-actin-Mash1-ires-GFP in the st13-14 chick neural tube induces Dll4 after 24 hours. Mash1 expression was confirmed by GFP immunolabelling (E) and Dll4 by ISH (E'). (F-G) Mash1 did not induce ectopic Chx10, but repressed endogenous Chx10 in the p2 domain. G is a magnified view of the ventral part of panel F. (H-H') Also, Mash1 did not induce ectopic V2b markers Gata2 or Scl. (J) Despite the fact that Mash1 is sufficient to induce Dll4 in chick (see E,E'), Mash1 is not required for Dll4 expression in mice; Dll4 is expressed as normal in the ventral spinal cord of Mash1-null mice.](image)
so, lose expression of Foxn4. Likewise, Scl and Dll4 transcripts were strongly downregulated compared with wild type at E11.5, consistent with their demonstrated dependence on Foxn4 (Fig. 7M,N and data not shown). Cre recombination is thought to be activated at or shortly before E10.5 in the Nestin-Cre line (Yang et al., 2006). In keeping with this, the morphology of the spinal cord was normal in the Nestin-Cre/Foxn4flHie at E11.5 (i.e., the ventral VZ was still present). Foxn4 and Dll4 were expressed at higher than normal levels in the mutant at E10.5, consistent with the idea that V2a/V2b progenitors are formed prematurely but have not yet had time to differentiate (Fig. 7G,H and data not shown). By contrast, and consistent with the above reasoning, Scl was expressed at a reduced level at E10.5 (Fig. 7K,L).

**DISCUSSION**

**Foxn4 activates V2b interneuron development**

We found that Foxn4 is both necessary and sufficient to activate Gata2, Scl and Gata3, suggesting that it is near or at the top of the genetic hierarchy that specifies V2b INs. This differs from our previous study, which found that co-electroporation of Foxn4 together with Mash1 was necessary to induce ectopic V2b gene expression, Foxn4 alone being insufficient (Li et al., 2005). At present we are unable to explain this difference but it could perhaps relate to differences in the level of Foxn4 expression achieved following electroporation. We have ruled out functional differences between the Foxn4 electroporation vectors used because in our hands both constructs give the result reported here. In any case, both our studies demonstrate that Foxn4 is a key determinant of the V2b sub-lineage.

It was shown previously that the transcription factor Scl is necessary and sufficient to induce V2b INs (Muroyama et al., 2005). Foxn4 transcripts are detected before Scl during normal development – at st13 in chick/E9.5 mouse, compared with st16-17 chick/E10.5 mouse (Li et al., 2005; Muroyama et al., 2005) (data not shown), suggesting that Foxn4 is upstream of Scl. Consistent with this, we have now shown that: (1) Scl expression is lost in Foxn4−/− mice, whereas Foxn4 expression is unaffected in Scl−/− mice; and (2) Foxn4 is able to induce Scl expression in chick electroporation experiments. Foxn4 induces robust expression of Gata2 in chick neural tube within 24 hours post-electroporation, whereas Scl and Gata3 are not detectable until 48 hours post-electroporation. This temporal order presumably reflects the fact that Gata2 is required for Gata3 expression (Karunaratne et al., 2002; Nardelli et al., 1999) and suggests that Gata2 is genetically upstream of Scl. This is backed up by the fact that Gata2 is expressed ahead of Scl during normal development in both chick and mouse (Muroyama et al., 2005) (data not shown). Gata3 expression is lost in Scl-null mice, placing Scl upstream of Gata3 (Muroyama et al., 2005). Taken together, the available data support a genetic cascade Foxn4 → Gata2 → Scl → Gata3. The reduction of Gata2 expression that was observed in Scl-null mice (Muroyama et al., 2005) can be attributed to loss of positive feedback from Gata3 (Karunaratne et al., 2002). A diagram of the proposed network is shown in Fig. 8.

**Foxn4 activates Dll4 and Mash1**

By loss- and gain-of-function experiments we found that Foxn4 is necessary and sufficient to activate Dll4 and Mash1 expression. We subsequently showed that Mash1 can also induce ectopic expression of Dll4 in chick spinal cord. This suggests that the conserved Mash1/Brn binding site in the Dll4 upstream region, reported by Castro et al. (Castro et al., 2006), is functional in vivo and further
suggested that Foxn4 might activateDll4 indirectly through Mash1. However, we found that Mash1 is not required for initiation of Dll4 expression in the mouse because Dll4 is expressed normally in the p2 domain of E10.5 Mash1-null spinal cord. It is possible that Mash1 might be required to maintain Dll4 expression after E10.5, but we have not examined older embryos. Alternatively, a requirement for Mash1 in the initiation of Dll4 expression might be masked in Mash1 mutant mice through compensatory upregulation of a related proneural factor such as Ngn1 (Neurog1) or Ngn2 (Neurog2). It is also possible that Foxn4 induces Dll4 directly; in endothelial cells, for example, Foxc1 and/or Foxc2 are known to activate Dll4 by binding directly to a Fox-binding site in the Dll4 gene upstream region (Seo et al., 2006).

Apart from regulating Dll4, Mash1 must have another role in promoting V2b IN fate, because Mash1-null mice at E10.5 are reported to have ~50% less V2b INs than normal (Li et al., 2005), despite the fact that Foxn4 and Dll4 are both expressed normally (Fig. 6D and data not shown). More work needs to be done to establish the precise role of Mash1 in V2b IN development.

**Notch1 is required for V2b interneuron development**

The connection between Foxn4, Dll4 and Mash1 led us to explore the role of Notch-Delta signalling more directly. We previously reported that when Notch1 function was disrupted in the ventral spinal cord, the result was a ~30% overproduction of Chx10, Lhx3) double-positive V2a INs and an ~18% loss of [Isl1 (Islet1), Lhx3) double-positive MNs, although the total number of Islet-positive MNs was unchanged (Yang et al., 2006). This was originally interpreted as a fate switch from MN to V2 IN production. However, in the present study we found that Gata3-positive V2b INs were completely lost, whereas the number of Hb9-positive MNs was not changed significantly in the Notch1 mutant. Therefore, we conclude that the increase in V2a INs is more likely to result from respecification of V2b INs than from respecification of MNs to V2a INs. Since the V2 phenotype of the conditional Notch1 mutant is analogous to that of the Foxn4-null mouse, it appears that both Notch1 and Foxn4 activities are required for V2b IN production.

The default behaviour of p2 progenitors in the absence of Notch1 or Foxn4 activity is to differentiate as V2a INs, suggesting that active Notch1 acts cell-autonomously in collaboration with Foxn4 to drive V2b development. We have not been able to address directly the question of whether Notch1 acts in a cell-autonomous fashion in V2b INs. However, we observed that electroporated Dll4 is co-expressed with endogenous Chx10 in some V2a INs (Fig. 5C), suggesting that Dll4-mediated inhibition of V2a INs is non-cell-autonomous, as expected from the classical view of Notch-Delta neighbour-to-neighbour signalling. This contrasts with Foxn4, which was never co-expressed with Chx10, in keeping with its expected cell-autonomous role. A cell-autonomous role for Notch is indicated by the requirement for presenilin 1 (Psen1) for V2b lineage development (Peng et al., 2007). Psen1 is involved in the intracellular cleavage of Notch (Wines-Samuelson and Shen, 2005). It also fits with the report that Notch1 binds to an enhancer in the upstream region of the Gata2 gene during hematopoiesis (Robert-Moreno et al., 2005).

Why does Dll4 electroporation inhibit V2a IN production without causing a compensatory increase in V2b INs in p2? Perhaps dll4 electroporation reduces the total number of V2 INs (V2a + V2b) by inhibiting production of V2 progenitors from their neuroepithelial precursors, while simultaneously biasing the fate of the remaining V2 progenitors from V2a towards V2b. If so, the fact that there is no significant change in the number of V2 INs in our electroporation experiments at st14-16 might be the result of two equal but opposing effects. If this explanation is correct, then the precise outcome of the experiment might depend critically on the time of electroporation, because this could alter the magnitude of one effect versus the other. Consistent with this idea, we found a small reduction in the number of V2b INs (as well as a reduction in V2a INs) when we electroporated at st11-12. Peng et al. (Peng et al., 2007) also found a reduction in total V2 INs in their electroporation experiments at st13. This model is necessarily speculative and other explanations are possible.

**Notch1/Dll4 signalling breaks symmetry and splits the V2 lineage**

What is the mode of action of Notch1 in V2 IN development? One possibility might be that p2 progenitors normally generate V2a INs first, before switching to V2b production, and that Dll4/Notch1 is needed to keep some progenitors in cycle long enough to generate V2b INs. In that case, eliminating Notch signalling might be expected to cause accelerated differentiation along the V2a pathway and loss of V2b differentiation, as observed. However, there is no evidence that V2a INs are formed before V2b INs. Chx10 and Gata3 are both expressed together for the first time at E10.5 in mouse (Liu et al., 1994; Nardelli et al., 1999). V2a and V2b subpopulations are formed simultaneously in chicken too (Karunaratne et al., 2002). We therefore propose that Notch1-Delta signalling has two consecutive or parallel functions in the p2 progenitor domain: (1) it inhibits neuroepithelial (radial) precursors from differentiating prematurely into V2 progenitors; and (2) it segregates V2 progenitors into V2a and V2b sub-lineages, inhibiting V2a and promoting V2b development.

The majority of cells that co-express Foxn4 and Dll4 are closely apposed pairs of cells at the ventricular surface, and these are likely to represent the products of recent progenitor cell divisions (Fig. 4A-
phenotype of V2b INs has not yet been established. It is possible that Notch-Delta signalling might be a general mechanism for creating complementary pairs of INs.

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Supplementary material
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