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Original Article

Notch activity is required to maintain floorplate identity and to control neurogenesis in the chick hindbrain and spinal cord

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ABSTRACT Notch signalling plays a major role in many invertebrate and vertebrate patterning systems. In this paper, we use high-titre, non-replicative pseudotype viruses to show that the two Notch ligands, Delta1 and Serrate1 (Jagged1), have differing activities in the developing chick spinal cord and hindbrain. In the walls of the neural tube, Serrate1 appears not to affect neurogenesis, in contrast to Delta1 which mediates lateral inhibition as elsewhere in the nervous system. In the floorplate we find that there is also a requirement for Notch, but with a different type of dependence on the two Notch ligands: cells with a floorplate character are lost when Notch activity is blocked with dominant-negative, truncated forms of either Delta1 or Serrate1. Our results are consistent with ligand-receptor specificity within the Notch signalling pathway, Serrate1 recognising selectively Notch2 (which is expressed in the floorplate), and Delta1 acting on both Notch2 and Notch1 (which is expressed in the walls of the neural tube).

KEY WORDS: *pseudotype retrovirus, neurogenesis, floorplate, chick, Notch*

Introduction

Neurons of the vertebrate CNS are mainly generated in the ventricular zone of the neural tube. Here, the neuroepithelium comprises a mixture of progenitor cells and newly born post-mitotic progeny that will migrate basally to become part of the mantle layer of differentiated neurons. These adopt a wide range of characters according to their positions and their times of birth (reviewed in McConnell, 1995; Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996; Livesey and Cepko, 2001). Specialised cells along the dorsal and ventral midlines of the neural tube, respectively forming its roofplate and floorplate, help pattern neurogenesis in the walls of the neural tube by producing signal molecules that establish intrinsic differences between progenitor cells in different positions (Tanabe and Jessell, 1996). The latter proliferate in a stem-cell-like manner for an extended period, producing vast numbers of neurons and glia of different types in an orderly pattern.

In both vertebrates and invertebrates, neurogenesis is regulated by lateral inhibition mediated by the Notch cell-signalling pathway, a process in which differentiating neural cells inhibit differentiation of adjacent uncommitted cells. Experiments in several model systems show that, in this way, Notch signalling maintains a balance between differentiating neural cells and proliferating progenitors, so that neurogenesis can continue (see below). Notch is a transmembrane receptor that is activated by the transmembrane ligands, Delta (Dl) and Serrate (Ser). Four *Notch* genes are known in mammals, along with three *Delta*s and two *Serrate*s (also called *Jagged* in mammals), while *Drosophila* has a single gene for each (reviewed in Muskavitch, 1994; Kimble and Simpson, 1997; Artavanis-Tsakonas *et al.*, 1999). Several of the vertebrate *Notch* homologues are expressed in the developing nervous system (Lindsell *et al.*, 1996), as are several *Dl* and *Ser* homologues, albeit less extensively and more transiently than Notch.

The role of Notch signalling in maintaining vertebrate neurogenesis is best characterised in the neural retina, in which *Notch1* is expressed throughout the ventricular zone of proliferating progenitors and newly generated neural cells. The latter transiently express *Delta1* (*Dl1*), activating *Notch1* in neighbouring progenitors that are thereby inhibited from commitment and differentiation (Austin *et al.*, 1995; Dorsky *et al.*, 1997; Henrique *et al.*, 1997; reviewed in Harris, 1997; for discussion of the similarities between neurogenesis in *Drosophila* and vertebrates, see Myat *et al.*, 1996; Henrique *et al.*, 1997). Negative feedback maintains the balance between neurons and progenitors, and sustains the process of neurogenesis. Thus, large-scale overexpression of *Dl1* in the retina of chick or *Xenopus* suppresses production of neurons, and drives cells to remain as progenitors, whereas blocking Notch signalling by means of a

Abbreviations used in this paper: CNS, Central Nervous System.

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dominant-negative truncated *Dl1* causes progenitors to exit the cell-cycle and to differentiate prematurely (Dorsky *et al.*, 1997; Henrique *et al.*, 1997). Lateral inhibition also operates elsewhere in the vertebrate CNS (Chitnis *et al.*, 1995; Wettstein *et al.*, 1997; Haddon *et al.*, 1998). Mutations in mice that block Notch signalling lead to an early excess of postmitotic prospective neurons in the embryonic mouse spinal cord, indicative of premature neuronal differentiation (Notch1, Su(H): de la Pompa *et al.*, 1997; Huppert *et al.*, 2000; Hes1: Ishibashi *et al.*, 1995; Presenilin: Handler *et al.*, 2000).

While Dl1 seems to be an essential part of the control mechanism in much of the CNS, some nascent neurons do not express this ligand. In two ventrolateral stripes in the hindbrain and spinal cord, *Dl1* transcripts are absent, and newly postmitotic cells – presumed to be nascent neurons – express instead the alternative Notch ligand, *Serrate1* (*Ser1,* Lindsell *et al.*, 1995; Myat *et al.*, 1996). This pattern of expression of *Ser1* and *Dl1* in complementary domains suggests that either ligand can mediate lateral inhibition. This possibility is supported by the expression pattern of Lunatic fringe (Lfng), an enzyme that glycosylates Notch and is thought to differentially modulate signalling by Dl and Ser (Bruckner *et al.*, 2000; Hicks *et al.*, 2000; Moloney *et al.*, 2000; Munro and Freeman, 2000). *Lfng* is absent from the *Ser1* stripes, but coexpressed with *Dl1* in the *Dl1*-expressing regions (Laufer *et al.*, 1997).

In contrast to the rest of the neural tube, floorplate cells divide only slowly and generate no neurons in the rostral spinal cord (Jessell and Dodd, 1990; Ericson *et al.,* 1997a; Placzek *et al.*, 2000). Nevertheless, Notch signalling may also play a continuing role there: floorplate cells express *Notch2,* which is absent from the neurogenic regions of the spinal cord and hindbrain (Lindsell *et al.*, 1996). Although no known Notch ligand is expressed within the floorplate, Ser1 is expressed in a pair of stripes that flank the floorplate (Lindsell *et al.*, 1996; Myat *et al.*, 1996).

To clarify the functions of Dl-Notch and Ser-Notch signalling in the spinal cord and hindbrain, we have used retroviral constructs to misexpress wild-type and dominant-negative forms of Dl1 and Serl in the neural tube. We find that Ser1, unlike Dl1, does not regulate neurogenesis. Nor does ectopic ligand expression affect the floorplate. However, dominant-negative versions of either Dl1 or Ser1 cause a loss of floorplate cells, either by cell death or by conversion into neurons. These results indicate that the Dl1 and Ser1 ligands have distinct activities, and reveal a novel function for the Notch pathway in the floorplate, perhaps mediated by Notch2.

Results

Efficient Non-Replicative Pseudotype Retroviruses for Gene Misexpression in the Chick Embryo

Previous experiments have assayed Dl1 function in chick embryos using replication-competent retroviruses (Logan *et al.*, 1996; Henrique *et al.*, 1997; Delfini *et al.*, 2000). However, the *Ser1* coding region is too large to be included in such vectors (e.g. RCAS; Hughes and Kosik, 1984; Fekete and Cepko, 1993) which have a maximum insert capacity of ~2.1kb. Also, the spreading infection caused by these viruses makes it difficult to determine exactly when a cell first experiences gene misexpression. We therefore generated defective pseudotype retroviruses that can accept much larger inserts because they lack endogenous viral genes required for replication. The viruses infect replicating, progenitor cells, but are unable to propagate further, and the number of misexpressing cells increases only by clonal expansion. Nevertheless, a high proportion of cells can be infected, because these viruses can be concentrated to high titres (Yee *et al.*, 1994).

We based our viruses on *LZRSpBNZ*, in which the Moloney Murine Leukaemia Virus (MoMLV) promoter drives transcription of the viral genome, and the nuclear replication and retention sequences of the Epstein-Barr virus help maintain stable episomes in the packaging cell line (Kinsella and Nolan, 1996). Preliminary experiments indicated that the MoMLV promoter is relatively inactive in chick embryos (unpublished), and so we introduced internally a 253 bp promoter fragment from the Rous Sarcoma Virus (RSV) to drive efficient transgene expression in the chick (Fig. 1, Hughes and Kosik, 1984). In most of our experiments, we included a downstream internal-ribosome-entry-site (IRES) followed by *GFP* to allow direct visualisation of infected cells (*RSVpBMN–G*; Fig. 1). These viruses should accept additional inserts of up to 6.5kb, well in excess of the 3.67kb coding sequence of *Ser1*. Insert capacity and viral titres (below) significantly exceeded those of the RSV-derived replication defective viruses described by (Chen *et al.*, 1999), which accept a maximal insert of 4.5kb.

Infectious particles were generated by cotransfection of plasmids encoding the viral construct and the VSV-G glycoprotein coat into the packaging cell line *293gp* which provides replication functions (Experimental Procedures, Yee *et al.*, 1994). Supernatant titres of 5.10^5-10^6 cfu/ml, concentrated to 5.10^8-10^9 cfu/ml, were sufficient to achieve extensive infection in the neural tube. The viruses appear genetically stable: 24h after infection with the *RSV-Dl1^T-IRES-GFP* (*RSVpBMN-Dl1^T-G*) retrovirus, all cells expressing GFP also express Dl1 (data not shown), indicating that GFP staining is a faithful marker of transgene expression.

Dl1-Notch Signalling regulates Neurogenesis in the Developing Spinal Cord

To validate the viruses, we tested the effects of misexpressing *Dl1* in the developing spinal cord, where the pattern of *Dl1* expression

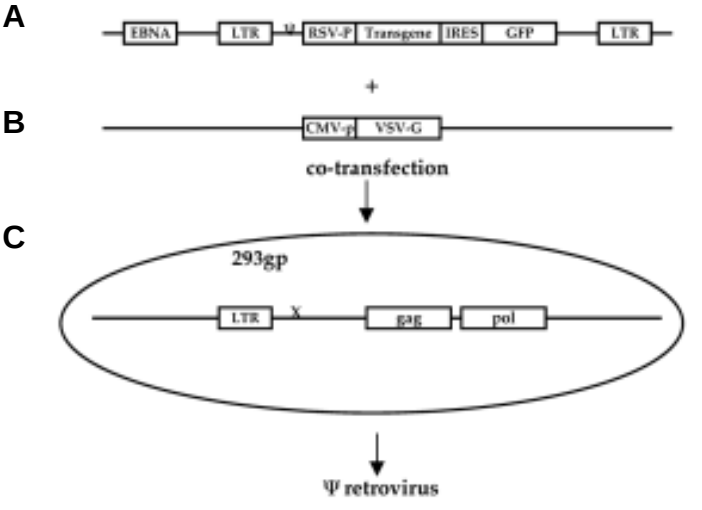


Fig. 1. Production of pseudotype retroviruses. *Pseudotype retroviruses were obtained after transient co-transfection of* RSVpBMN *or* RSVpBMN-G **(A)** *viral vectors and a plasmid expressing the VSV-G glycoprotein coat* **(B)** *into the packaging cell line* 293gp **(C)**. *EBNA refers to nuclear replication and retention sequences of the Epstein-Barr virus. ψ indicates the encapsidation site which has been mutated (X) in the* 293gp *packaging cells.*

hints strongly at a role in regulating neurogenesis. We first examined expression of Dl1 protein, using a polyclonal antibody directed against an extracellular epitope of chick Dl1 (Henrique *et al.*, 1997, Experimental Procedures). Dl1 expression parallels that of *Dl1* transcripts (Henrique *et al.*, 1995; Myat *et al.*, 1996), being detectable throughout the length of the spinal cord and hindbrain, except for two narrow ventral stripes on each wall of the neural tube and the floorplate (Fig. 2A; Myat *et al.*, 1996; Laufer *et al.*, 1997). Staining with a polyclonal antibody directed against an intracellular epitope of chick Ser1 (Adam *et al.*, 1998; Eddison *et al.*, 2000; Experimental Procedures) confirms that the stripes within which Dl1 expression is absent correspond to the Ser1-expressing stripes (Fig. 4A, Myat *et al.*, 1996). Dl1 staining is restricted to scattered cells whose positions and lack of BrdU staining (Myat *et al.*, 1996) indicate that they are newborn postmitotic neural cells, about to migrate basally into the mantle layer. The distribution of Dl1 antigen, like that of Dl in *Drosophila* (Parks *et al.*, 1995; Lai *et al.*, 2001; Pavlopoulos *et al.*, 2001) and in zebrafish (Itoh *et al.*, 2003) is punctate (Fig. 2B), suggesting that this protein is mostly concentrated in cytoplasmic vesicles. Many of the ligand-expressing cells, whose bodies lie in the ventricular zone, have processes labelled in this manner and extending all the way out to the pial (basal) surface of the neural tube (Fig. 2A).

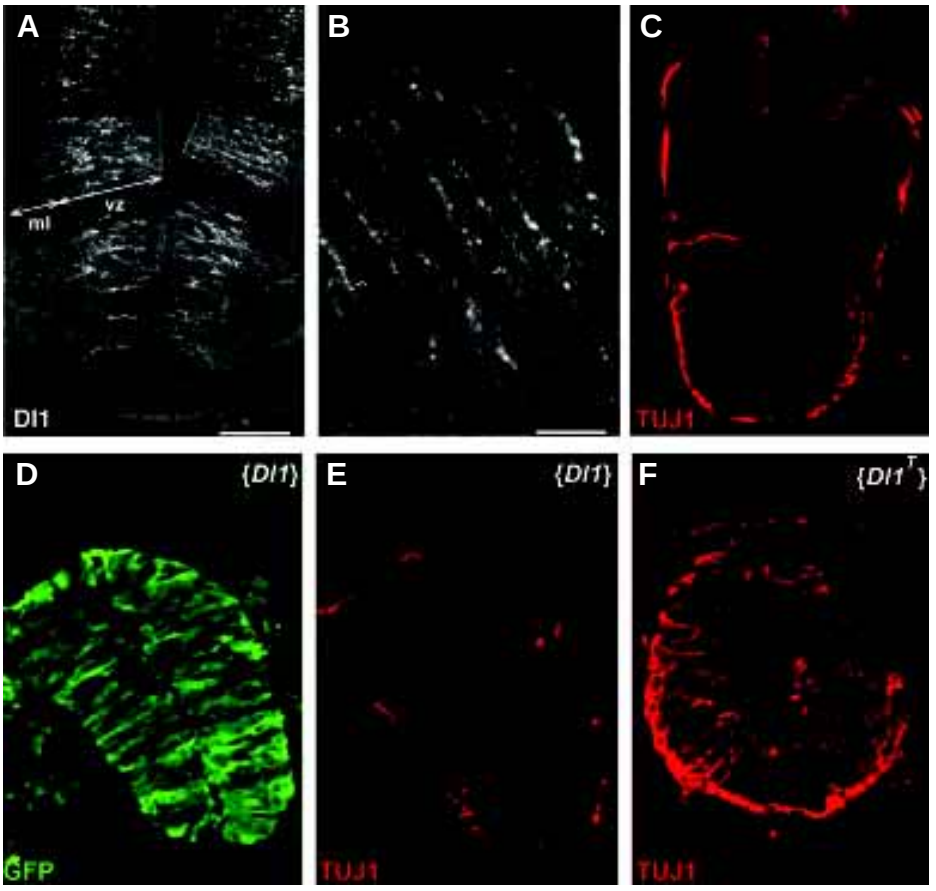


Fig. 2. Dl1 regulates neurogenesis in the rostral spinal cord and hindbrain. (A,B) *Dl1 protein expression in rostral spinal cord in stage 22/23 (E4) embryo. Dl1 accumulates predominantly in a punctate manner, presumably in vesicles (B), within a subset of neural progenitors whose cell bodies are located towards the edge of the ventricular zone (A). Levels of Dl1 expression in the Ser1-expressing domain adjacent to the floorplate appear lower than elsewhere in the neurogenic neural tube (panel A and Myat* et al., *1996).* **(C-F)** *Transverse sections of spinal cord (stage 17/18, E3). Compared to uninfected embryo (C), TUJ1 expression (Lee* et al., *1990) is depressed in embryos infected with* RSV-Dl1-GFP *(D,E; same embryo; n=8 embryos), and enhanced in* RSV-Dl1^{T}-GFP-*infected embryos (F; n=10). In these and subsequent figures,* {Dl1}, {Dl1^{T}}, *etc. refer to viral genotype and thus, the transgene being monitored by GFP expression (green in panel D). Dorsal is to the top; vz, ventricular zone; ml, mantle layer; scale bars, (A) 100μm; (B) 33 μm.*

To test the effects of misexpressing *Dl1*, we injected pseudotype retroviruses (*RSVpBMN-Dl1-G*) into the lumen of the neural tube of stage 10/12 (E1.5-2) embryos, such that expression of exogenous protein is first detected at about the time the longitudinal stripes of *Dl1* and *Ser1* appear (stage 17/18; E3; data not shown). Artificial overexpression of *Dl1* inhibits neural differentiation, consistent with the results previously obtained in the neural retina (Henrique *et al.*, 1997). Large patches of apparently contiguous Dl1-positive cells are evident as early as 30h (E3) post-infection (e.g. Fig. 2D). Neurogenesis is depressed in infected patches. This is clearly shown, for example, by reduced immunostaining for the motoneuron marker, Islet1/2(cf. Fig. 3 A,B), and is also evident in sections stained for the pan-neural TUJ1 antigen (βIII-tubulin; Fig. 2 D,E). Thus ectopic Notch signalling inhibits neurogenesis in the spinal cord and hindbrain.

To show that this result reflects a normal role for Notch signalling, we injected viruses expressing a truncated Dl1 molecule lacking most of the intracellular domain (Dl1^{T}, previously called Dl1^{Stu}, Chitnis *et al.*, 1995; Henrique *et al.*, 1997). This behaves as a dominant-negative protein which blocks Notch signalling in a cell-autonomous fashion – signal reception is blocked in the cell that expresses the truncated ligand (Henrique *et al.*, 1997). Spinal cord cells infected with either *Dl1^{T}* or *Dl1^{T}-GFP* virus differentiate as neurons in great excess (Fig. 3 C,D). The epithelial organisation of the neural tube is severely disrupted (Fig. 2F and not shown), as expected if all cells differentiate into neurons, leaving no progenitors or glia to maintain epithelial architecture.

Spinal Cord Neurogenesis is unaffected by Overexpression of Ser1 or Ser1^{T}

Ser1 is expressed in three stripe domains on each wall of the neural tube. In two of these, the domains from which Dl1 expression is excluded, Ser1 is expressed in scattered cells that appear from their locations to be nascent neurons (Fig. 4A; Myat *et al.*, 1996). The situation is different in the third pair of stripes, flanking the floorplate. Ser1 expression in these last domains differs in several respects: it is less intense, and is seen in most or all cells within the domain, both in ventricular zone cells and in differentiated cells expressing the motoneuron marker, Islet1/2 (Fig. 4 B,B'). Moreover, protein staining is relatively uniform, not punctate as in the other Ser1 stripes and for

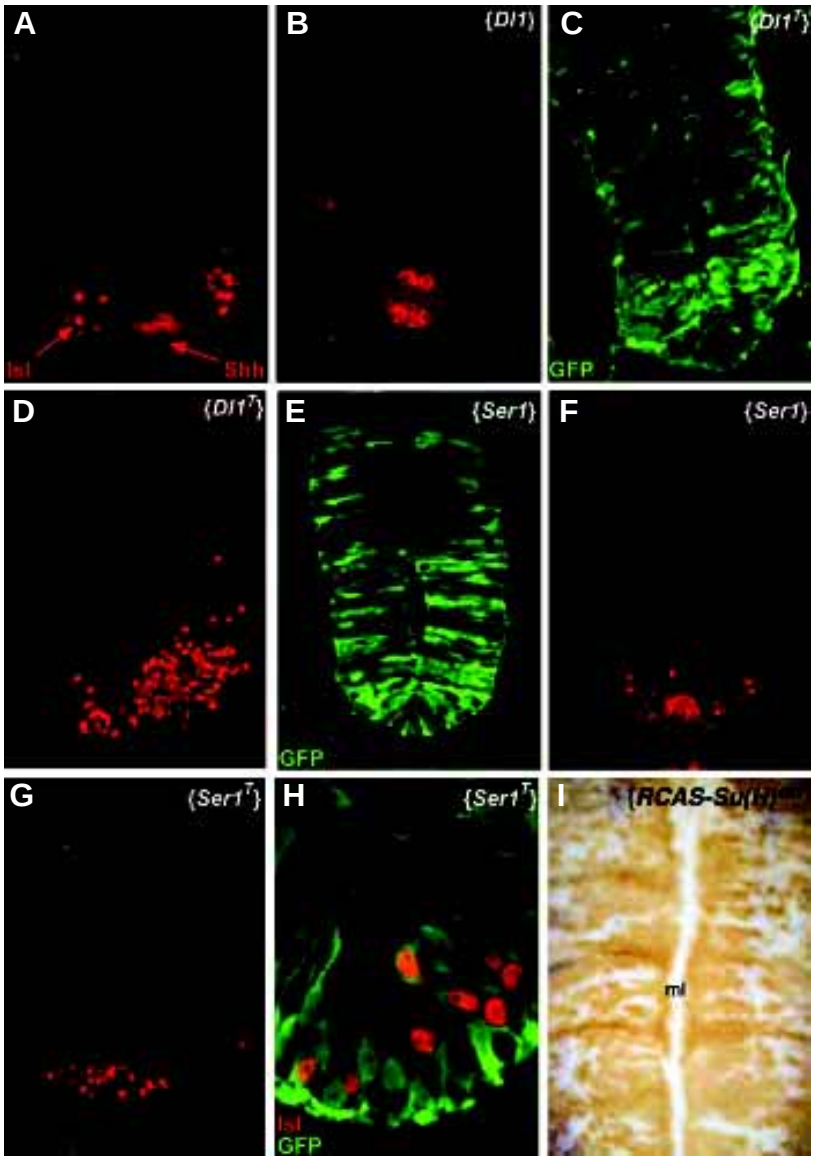


Fig. 3. Interference with Dl1 signalling causes widespread disturbance of Islet1/2 expression and neuroepithelial integrity, while interference with Ser1 signalling affects only the floorplate. *Embryos were infected with pseudotype virus carrying* Dl1-GFP **(B)**, Dl1^T-GFP **(C,D),** Ser1-GFP **(E,F)** *or* Ser1^T-GFP **(G,H)**, *and examined at stages 17/18 (E3). These were stained for GFP (green; C,E,H) to visualise infection, and for Islet1/2 (nuclear red) and Shh (cytoplasmic red) to visualise motoneurons (Ericson* et al., *1992) and floorplate cells (Echelard* et al., *1993), respectively. Ectopic Dl1 inhibits Islet1/2 expression (B) compared with uninfected embryos* **(A)**; *Shh expression is unaffected. Dl1^T leads to ectopic Islet1/2 expression (C,D; same embryo). Islet1/2 expression flanking the floorplate is unaffected by Ser1 (E,F; same embryo) or Ser1^T (G), and the partitioning of infected cells between the proliferative ventricular zone or differentiating mantle layer is unaffected (E). In the floorplate itself, however, Ser1^T causes ectopic Islet1/2 expression, even in individual cells, and reduces Shh expression (G,H).Dorsal view of an E4 flat mounted spinal cord heavily infected with an RCAS virus* (RCAS-Su(H)dn), *showing that midline floorplate cells are refractory to spreading infection, even though infectable by the replication-defective pseudotype viruses. Brown stain is antibody against viral Gag-p27 protein. ml, midline.*

Dl1 (Fig. 4 A,B). These differences in protein presentation might be indicative of a different function for Ser1 in this domain (see Discussion).

To assay Ser1's activity during neurogenesis, we expressed it or a version lacking most of the intracellular domain (Ser1^T), using the pseudotype viruses. Neither construct appears to affect production of neurons in the neurogenic regions of the neural tube, although Ser1^T has effects in the floorplate (see below). Cells within patches of cells infected with either construct can differentiate into neurons expressing Islet1/2 (Fig. 3 E-H) and TUJ1 (Fig. 4 C,D), and can be found in the mantle layer (Fig. 3E and data not shown). Expression of the neural progenitor marker Pax6 (Ericson *et al.*, 1997b) appears normal (data not shown). 2 days post-infection (E4), infected cells are present both in the ventricular zone and mantle layer, indicating that the capacity of both Ser1- and Ser1^T- misexpressing cells to differentiate is unaffected (Fig. 4 E,F). The apparent lack of Ser1 activity is unlikely to be due to a damaged viral construct because the same full-length Ser1 construct perturbs immune responses *in vitro* and *in vivo* (Hoyne *et al.*, 2000), and the equivalent truncated Ser1^T virus is active in the floorplate (see below). Although we cannot exclude the possibility that Ser1 exerts subtle effects on neural cell-fate decisions, these results clearly indicate that Ser1 and Dl1 are not interchangeable during vertebrate neurogenesis, at least within the Dl1 domains.

Dl1 and Ser1 might have different activities because the latter cannot act where the Notch1 receptor is modified by the Lfng glycosyl transferase (Bruckner *et al.*, 2000; Hicks *et al.*, 2000; Moloney *et al.*, 2000; Munro and Freeman, 2000), i.e. in the *Dl1* domains. If so, artificial misexpression of Ser1 should have powerful effects on neurogenesis in the Ser1 stripes, where Lfng is not expressed. The Ser1 stripes are narrower than the Dl1 domains, making it more difficult to determine if they are selectively affected by Ser1 or Ser1^T. Nevertheless, they too appear insensitive to Ser1 or Ser1^T expression. Neither virus causes obvious deficiency or excess of neurons or progenitor cells in these stripes (Fig. 4 E,F), and infected cells are present throughout the thickness of the walls of the neural tube (e.g. Figs. 3E, 4 E,F). Expression of endogenous *Ser1* and of *lfng*, which marks the Ser1 domains, appears not to be disrupted (Fig. 4 E,F). It seems unlikely, therefore, that the lack of effect of *Ser1* misexpression is simply a reflection of the pattern of *Lfng* expression.

As a further test of this idea, we examined the consequences of misexpressing *Lfng*. If (as in *Drosophila*) Ser1 is only effective as a Notch ligand in the absence of Lfng, ectopic expression of Lfng should lead to a disturbed pattern of neurogenesis within the Ser1-expressing domains. We drove general expression of *Lfng* in the developing spinal cord using a replication-competent retrovirus virus (*RCAS-Lfng*; Laufer *et al.*, 1997) and analysed *Ser1* expression and Islet1/2 expression as markers of neurogenesis in the target domains. *Lfng* causes no detectable alteration in *Ser1* expression (Fig. 4G) or Islet1/2 expression in the *Ser1* domains (Fig. 4h), arguing that the failure of *Ser1* to regulate neurogenesis is not due to *Lfng* alone. In any case, these results clearly show that Ser1 and Dl1 are not interchangeable during vertebrate neurogenesis.

Blocking Notch Signalling induces Expression of Neural Markers in Floorplate Cells

While most of the spinal cord is dedicated to neurogenesis, its ventralmost cells – those of the floorplate – are relatively mitotically quiescent and do not generate neurons. Floorplate cells selectively express Notch2 and much reduced levels of Notch1 (Fig. 5A; Lindsell *et al.*, 1996), but do not express detectable levels of any known Notch ligands. In specimens heavily infected with replication-competent RCAS virus, the central cells of the floorplate stand out as refractory to spreading infection (Fig. 3I), presumably because they are non-dividing.

Nevertheless, we find that our replication-defective pseudotype virus can infect the floorplate cells (see Figs. 2D and 3 E,H). When we use this as a vector to drive expression of Dl1^T in the floorplate, the fate of the cells is drastically altered. Infected embryos show ectopic expression of Islet1/2 and TUJ1 at the midline, and the normal epithelial organisation of the floorplate region is disrupted (Fig. 5B).

To check whether disrupting Notch signalling causes loss of floorplate-specific markers, we examined expression of the winged-helix transcription factor HNF3β, which is expressed strongly in the floorplate and only weakly in the adjacent cells (Ruiz i Altaba *et al.*, 1995). 30h post-infection, the number of HNF3β-expressing cells in the ventral midline region decreases from 33.8 cells per section of uninfected spinal cord (±5.8; n=14) to 17.7 (±4.7; n=18) in infected sections, indicating that the appearance of ectopic neural cells is accompanied by partial loss of floorplate cells (cf. Fig. 6 C,C',E). Residual HNF3β expressing cells, typically lying in the most central part of the floorplate region, may not have been infected. Expression of Shh also seems to be reduced in infected embryos, although it is not easy to quantify the numbers of cells affected because the protein is not nuclear (data not shown).

Transformation in the fate of infected cells in the floorplate is also evident in the severe disruption of its morphology, perhaps reflecting a loss of cell adhesion. The heavily infected floorplate is thinner and wider than in control embryos (Fig. 5B). TUNEL staining did not reveal extensive death of infected floorplate cells, at least between E3 and E5 (Fig. 6F), although this could be because dying cells are rapidly cleared.

Strikingly, Ser1^T has effects on floorplate development similar to those of Dl1^T. Infected cells in the ventral midline express the neural markers TUJ1 and Islet1/2 (Figs. 3 G,H; 5B). Correspondingly, the number of HNF3β-expressing cells at E3 per section is reduced from a control value of 33.8±5.8 to 14.5 ±5.3 (n=10) in Ser1^T-infected

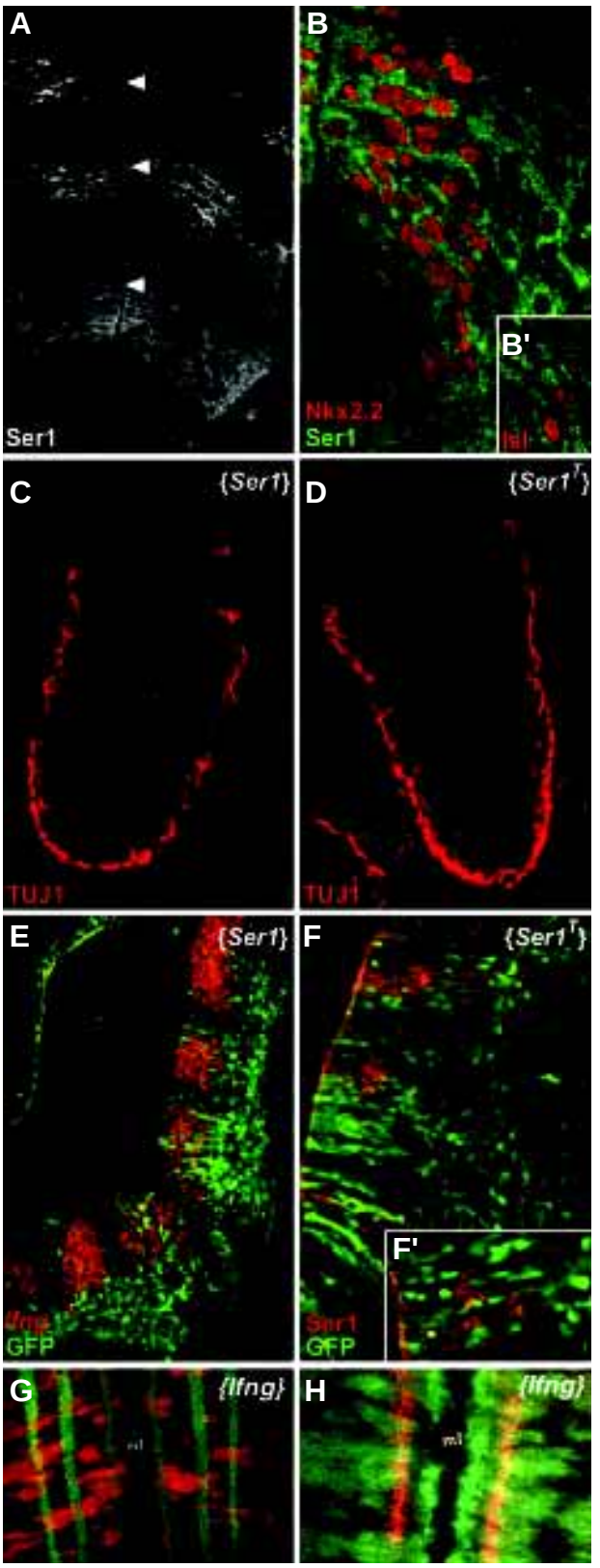
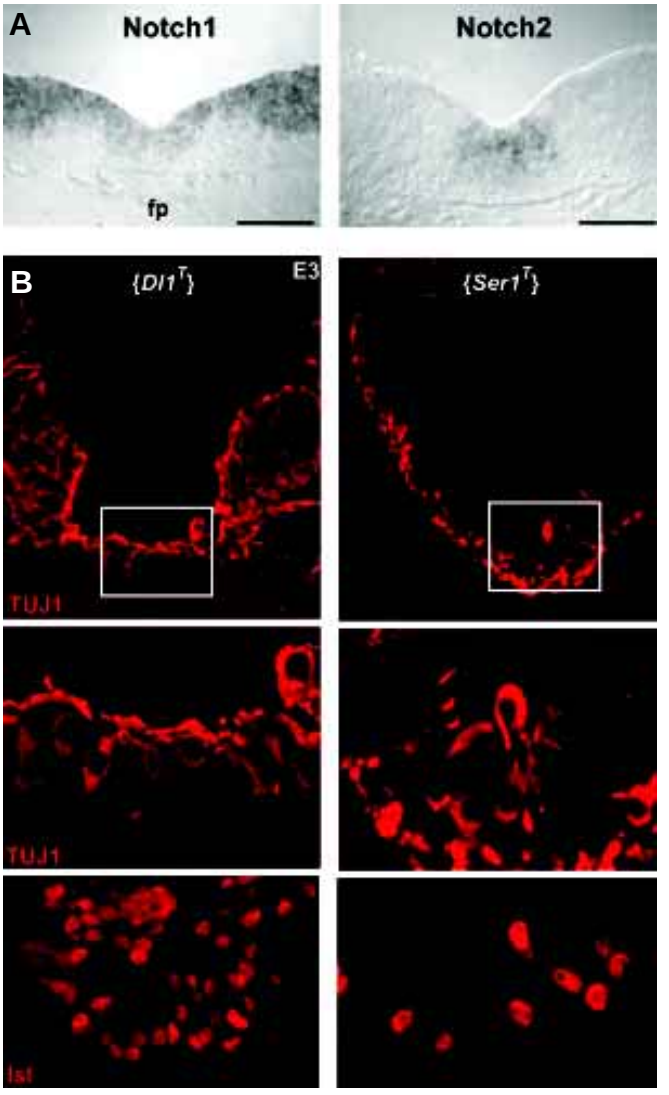


Fig. 4. Neurogenesis in the walls of the neural tube is unaffected by overexpression of Ser1, Ser1^T or Lfng. (A) *Transverse section of E4 rostral spinal cord showing that Ser1 protein is expressed in three stripes (arrowheads), complementary to the domains of Dl1 (cf. Fig 2A). In the two upper stripes, Ser1 accumulates in scattered cells in a punctate manner, presumably in vesicles.* **(B,B')** *Ser1 (green) expression in the third stripe is more general, extending beyond the ventricular zone (B; Nkx2.2-expressing cells, red), and being also detected in Islet1/2-expressing neurons (inset B'; Islet1/2, red). (C-F) Transverse sections of spinal cord infected with* Ser1-GFP **(C,E)** *or* Ser1^T-GFP **(D,F,F')** *and fixed at stages 17/18 (E3; C-D) or 22/23 (E4; E,F,F'). Expression of the neuronal marker TUJ1 (Ser1, n=2; Ser1^T, n=9) (red, C,D) appears normal following the extensive ectopic expression of Ser1 or Ser1^T. (F) Dorsoventral patterning is unaffected by expression of either Ser1 (E) or Ser1^T (F), as judged by the domains of* Lfng *expression (E; red; n=2), or the stripes of endogenous Ser1 (red) protein (F,F'; n=2).The inset (F') corresponds to high magnification picture in (F). In panels B,E,F, the ventricular zone is to the left of the neuroepithelium.* **(G,H)** *Dorsal views of flat-mounted spinal cords 2 days after infection with* RCAS-Lfng *(stage 22/23; E4). Infection was monitored by immunofluorescence against Gag-p27 (G, red; H, green) of the RCAS virus.* Ser1*-expressing nascent (G, green) and Islet1/2-expressing differentiating neurons (H, red) are unaffected by misexpression of* Lfng.



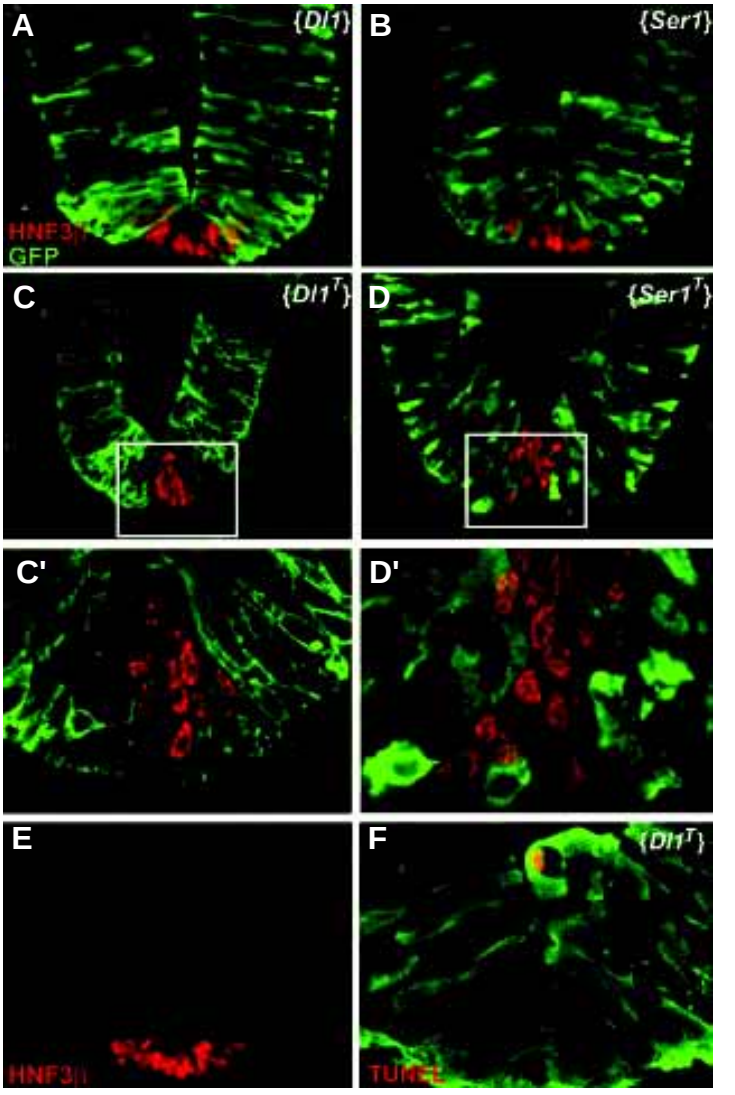


Fig. 5. (Left) Dominant-negative Notch ligands cause floorplate cells to express neural markers. (A) *Expression of* Notch1 *and* Notch2 *transcripts in the hindbrain at stage 22/23 (E4).* Notch1 *is expressed throughout the ventricular zone and weakly in the floorplate, while expression of Notch2 is restricted to the floorplate. Scale bars in A,B, 125 μm.* **(B)** *Immunofluorescence of transverse sections of rostral spinal cord and hindbrain infected with* Dl1^{T}-GFP *(left column) or* Ser1^{T}-GFP *(right column). Expression of TUJ1 (top two rows; E3) or Islet1/2 (bottom row; E4) indicates cells of neuronal character. White boxes in top row indicate the frame of the corresponding high magnification pictures in the row below. See also Fig. 2 G,H. fp, floor plate.*

Fig. 6. (Right) Interfering with Notch signalling reduces expression of the floorplate marker HNF3β. *Immunofluorescence on transverse spinal cord sections at E3, using antibodies to GFP (green, A,B,D,D'), Dl1 (green, C,C') and HNF3β (red).* **(A,B)** *Extensive ectopic expression of Dl1 (A; n=3) or Ser1 (B; n=4) does not affect the number or distribution of HNF3β -expressing cells.* **(C,C')** *Overexpression of Dl1^{T} leads to a disruption of the neural tube including the floorplate. HNF3β is still expressed, although in fewer cells (n=9) than in wild-type embryos* **(E). (D,D')** *Ser1^{T} overexpression in the ventral tube leads to a reduction in the number of HNF3β-expressing cells. Most of the remaining HNF3β–expressing cells do not express Ser1^{T} (n=7). The white boxes in (C,D) indicate the frame of the corresponding high magnification pictures (C',D').* **(F)** *TUNEL staining (red) of apoptotic cells on transverse spinal cord sections after heavy infection with* Dl1T *(E4; GFP, green); only one TUNEL-positive cell is visible here, and it is rare to see more.*

sections. Together, these phenotypes indicate that Ser1^{T} has a similar activity to Dl1^{T} in the floorplate, causing cells to differentiate as neurons (or perhaps to die and be replaced by neurons), while differing from Dl1^{T} outside the floorplate, where Ser1^{T} fails to cause excessive neuronal differentiation.

The above results suggest that Notch activity is required to maintain normal organisation of the floorplate and to prevent its cells from differentiating into neurons. To test whether floorplate cells are sensitive to overactivation of Notch signalling, we examined the effects of ectopic expression of full-length Dl1 and Ser1. The epithelial morphology and patterns of gene expression appear unaffected in infected floorplate cells, which continue to express the markers Shh and HNF3β (Figs. 3 B,E,F, 6 A,B,E), and there is no noticeable expansion of the region expressing these markers. Thus, ectopic expression of normal, functional Notch ligands has no effect on floorplate development.

Discussion

In this paper, our experiments using high-titre pseudotype viruses have identified an unexpected role for Notch signalling in maintaining the floorplate in the chick hindbrain and spinal cord. Floorplate cells do not normally contribute to neurogenesis, although they play a crucial role in organising and patterning the neural tube and guiding axon outgrowth. However, inhibiting Notch signalling in the floorplate region with Dl1^T or Ser1^T causes a loss of cells expressing the floorplate marker HNF3β, and in their place we see neurons. These results suggest that floorplate cells have a latent competence for neuronal differentiation and that Notch signalling is required to prevent them from adopting a neuronal fate.

An alternative interpretation is that floorplate cells infected with Dl1^T or Ser1^T virus die and disappear, allowing adjacent Islet1/2 expressing neurons to infiltrate the midline region. We do not detect substantial numbers of TUNEL-positive cells at relevant stages (Fig. 6F), although dead or dying cells could be cleared rapidly. In any case, we can conclude that the fate of floorplate cells is altered by blockade of Notch signalling.

Previous work in the zebrafish has shown that Delta-Notch signalling controls the allocation of cells at the time of gastrulation to become floorplate as opposed to notochord or hypochord (Appel *et al.*, 1999; Donoviel *et al.*, 1999). Our present findings in the chick concern a different, later function: we interfere with Notch signalling effectively only from stage 16/17, well after the chick floorplate is established (stage 10), and so the consequences we see reflect a novel role for Notch in floorplate maintenance rather than specification

Our findings also clarify the role of Notch signalling during neurogenesis in the walls of the neural tube. We have demonstrated that production of differentiated neurons in these parts of the developing CNS is controlled by Delta-Notch-mediated lateral inhibition, as in the *Xenopus* and zebrafish primary nervous system and the neural retina (Chitnis *et al.*, 1995; Dorsky *et al.*, 1997; Henrique *et al.*, 1997; Haddon *et al.*, 1998). Ectopic Dl1 expression blocks differentiation of progenitor cells, and inhibition of Notch signalling with the truncated Dl1^T protein causes progenitors to differentiate (Figs. 2,3). As in the neural plate and retina, and probably throughout the CNS, lateral inhibition via Dl1 signalling regulates the balance between neural progenitors and differentiated neural cells. These results are consistent with those from mice mutant in the Notch signalling pathway, which display early overproduction of neurons (Ishibashi *et al.*, 1995; de la Pompa *et al.*, 1997; Handler *et al.*, 2000; Huppert *et al.*, 2000).

In contrast with Dl1, we have no evidence that Ser1 is active in lateral inhibition in the neural tube even though, like Dl1, it is expressed transiently in subsets of postmitotic cells whose locations suggest that they are nascent neurons. Outside the floorplate, neither Ser1 nor Ser1^T virus has any obvious effects on proliferation or differentiation in the hindbrain and spinal cord (Figs. 3 E-H, 4). Thus, in embryos infected with Ser1^T virus, we still see an approximately normal number of progenitor cells in the stripes where Ser1 is normally expressed (Fig. 4F). Nevertheless, it would be difficult to detect mild disturbances in the narrow Ser1 stripes, and we cannot exclude the possibility that a subpopulation of progenitors is sensitive to Ser1 signalling, or that progenitors from outside the stripes can compensate for defects within the stripes.

Ser1 might have some function other than lateral inhibition within the walls of the neural tube. For example, as a cell-surface molecule, it might help to guide the formation of longitudinal fibre tracts and segregate functionally distinct classes of longitudinal axons to different dorsoventral levels. Indeed, antibody staining reveals that Ser1 protein is present in cell processes that span the full thickness of the wall of the neural tube in the Ser1 stripe regions (Fig. 4A), and Notch signalling has effects on cell movement and guidance in flies and vertebrates (Franklin *et al.*, 1999; Sestan *et al.*, 1999; Redmond *et al.*, 2000). Detailed analysis of neuronal pathfinding is needed to test these ideas.

Why might neural progenitor cells be unresponsive to Ser1? Ser1 may be poorly recognised by Notch1, the predominant receptor in the neurogenic epithelium and the receptor which presumably mediates lateral inhibition there (de la Pompa *et al.*, 1997). This explanation is consistent with work showing that Ser1 binds poorly to Notch1 *in vitro* (Shimizu *et al.*, 1999). A second potential factor is post-translational modification of Notch receptor protein. Biochemical analysis has shown that *Drosophila* and vertebrate Fng proteins are glycosyl transferases that modify Notch and enhance its sensitivity to Dl binding and signalling (Bruckner *et al.*, 2000; Hicks *et al.*, 2000; Moloney *et al.*, 2000; Munro and Freeman, 2000). *In vivo*, *Drosophila* Fng acts cell-autonomously to potentiate sensitivity to Dl signalling and to reduce sensitivity to Ser signalling (Panin *et al.*, 1997). In the vertebrate CNS, Lfng is expressed in the same broad domains as Dl1, and so could prevent activation of Notch1 by ectopically expressed Ser1, in these regions at least. However, this is not likely to be the key factor that makes neural progenitors unresponsive to Ser1 because widespread misexpression of *Lfng* in the spinal cord does not perceptibly affect neurogenesis in the *Ser1*-expressing domains (Fig. 4 G,H).

An obvious suggestion is that Ser1, in conjunction with Lfng, organises tissue patterning by provoking special types of cell behaviour at the boundaries of the gene expression domains, as Ser in conjunction with Fringe does in the developing *Drosophila* wing (Couso *et al.*, 1995; Diaz-Benjumea and Cohen, 1995; Doherty *et al.*, 1996). This seems unlikely with respect to the side-walls of the neural tube, however, since the pattern of Ser1 and Delta1 stripes in these regions appears not to be affected by misexpression of either Ser1 or Lfng. The Drosophila analogy seems weak in any case for the two more dorsal stripes, where Ser1 is expressed transiently in scattered cells and in domains complementary to those of Lfng; whereas at the *Drosophila* wing margin, Ser is expressed in contiguous cells together with Fringe (Panin *et al.*, 1997). Such a boundary role is perhaps more plausible for the ventral-most stripes, adjacent to the floorplate, which differ from the others in that Ser1 is expressed diffusely in all cells. It is conceivable that these cells next to the floorplate, expressing both Ser1 and Lfng, might activate Notch in the neighbouring floorplate cells, which express neither Ser1 nor Lfng.

Truncated Notch ligands, such as Dl1^T and probably Ser1^T, make cells that express them refractory to Notch activation by ligands produced by neighbouring cells (Henrique *et al.*, 1997). Both these constructs appear to alter the fate of floorplate cells that express them. Ser1^T, in particular, has strong effects in the floorplate even though both it and ectopic Ser1 produce no visible effects on Dl1-Notch1 signalling or neurogenesis in the walls of the neural tube. Together, these results suggest that Notch signalling in the floorplate is mediated by a receptor that is sensitive to blockade by Ser1^T and thus different from the type of Notch protein directing lateral inhibition in the walls of the neural tube, which is insensitive to both Ser1^T and Ser1. Notch2 is a prime candidate for such a role: it is selectively expressed in the floorplate (Fig. 5A; Lindsell *et al.*, 1996), whereas

Notch1 is preferentially expressed in the ventricular zone. Selective interaction of Ser1^T with Notch2 is consistent with the *in vitro* binding preference of Ser1 for Notch2 over Notch1 (Shimizu *et al.*, 1999).

The homozygous phenotypes of two distinct Notch2 mutants have been studied in the mouse – one a hypomorph that survives to birth (McCright *et al.*, 2001), the other probably a functional null, dying at E11.5 (Hamada *et al.*, 1999). The latter paper mentions that the floorplate appears normal at E10.5, although later stages are not examined. However, analysis of chimaeric mice containing mutant Notch2 cells has provided evidence that this gene is essential in the mouse brain for normal development of the roofplate, a structure somewhat similar to the floorplate (Kadokawa and Marunouchi, 2002). There may be a real difference between mouse and chick in the requirement for Notch2 in the floorplate, even though both species express the gene there. It is possible, for example, that the two organisms differ in the degree of redundancy between Notch2 and Notch1 in the floorplate: residual Notch1 activity might substitute adequately for Notch2 activity in the mouse but not in the chick. There are, of course, many parallels for species differences of this type.

The source and character of the endogenous ligand acting on Notch in the floorplate are unclear. We have already suggested one possibility: the ligand could be Ser1, expressed in the cells immediately adjacent to the floorplate. This could have an effect throughout the floorplate in at least two ways: the flanking cells might send filopodia or other processes into the floorplate region (Ramirez-Weber and Kornberg 1999); or these cells might secrete diffusible Ser1. Notch ligands are conventionally thought to be attached to the cell surface, but some evidence indicates they may also act as diffusible extracellular fragments (Couso *et al.*, 1995; Qi *et al.*, 1999; Hicks *et al.*, 2000; Morrison *et al.*, 2000). However, the activities of such cleavage products are unclear, and vary according to the assay system. *In vivo*, soluble extracellular Notch ligands have weak dominant-negative activity in *Drosophila* (Hukriede *et al.*, 1997; Sun and Artavanis-Tsakonas, 1997).

While a role for Ser1 from cells flanking the floorplate is attractive, it is also possible that Notch2 could be activated by a floorplate-derived ligand that is either novel or expressed at levels below our threshold of detection. A further possibility is that Notch2 is constitutively active in the floorplate in a ligand-independent fashion, perhaps weakly, but to an extent sufficient to block neuronal differentiation there, and in such a way that the constitutive activity is sensitive to blocking by Dl1^T and Ser1^T.

Regardless of which of these alternatives is correct, our observations, using high-titre, non-replicative pseudotype viruses for gene misexpression in the chick, clearly point to a novel function for Notch signalling in the floorplate, and show that the function of Notch in the walls of the neural tube is differently regulated by the two ligands Ser1 and Delta1.

Materials and Methods

RSV Plasmids

The viral vector (*RSVpBMN*) was generated from *LZRSpMNZ* (Kinsella and Nolan, 1996) by amplifying 253bp from the enhancer of the Rous Sarcoma Virus (Kandala and Guntaka, 1994) as a *BglII-BamHI* fragment by PCR using the following primers:

GTAATCAGATCTAATGTAGTCTTATGCAATACTCCTGTAGTCTTG (5')
and
GTAATCGGATCCTGTGGTGAATGGTAAAATGGC**G**T**C**TATTGTATCG (3')

(Bold letters designate point mutations in the polyadenylation signal). The viral vectors *RSV-Dl1, RSV-Dl1^T, RSV-Dl1-IRES-GFP, RSV-Dl1^T-IRES-GFP, RSV-Ser1-IRES-GFP, RSV-Ser1^T-IRES-GFP* were prepared via *RSVpBMN* and an intermediate viral vector, *pBabe-puro* (Morgenstern and Land, 1990). To generate *RSV-Dl1* and *RSV-Dl1^T*, *BamHI*, blunt ended-*EcoRI* fragments from *Dl1-pBabe puro* and *Dl1^T-pBabe puro* (mouse *Delta1*, Lowell *et al.*, 2000) were cloned into *BamHI* blunted-*NotI RSVpBMN* plasmid. To make intermediate *Ser1* and *Ser1^T* vectors, PCR was used to generate fragments encoding full length human *Ser1* (amino acids 1-1222) and truncated *human-Ser1^T* (amino acids 1-1102) as *BamHI-EcoRI* fragments, and cloned into *BamHI-EcoRI pBabe-puro*.

IRES-GFP constructs were generated in 3 steps. First, an *EcoRI-SalI IRES-GFP* fragment from the *MIGR1* plasmid (Pear *et al.*, 1998) was cloned into the *EcoRI-Sal1* site of each of the four *pBabe-puro* constructs. Second, downstream *HindIII* and *Not1* sites were introduced by subcloning *BamHI-SalI* fragments of these new constructs (*Dl1-IRES-GFP*, *Dl1T-IRES-GFP*, *Ser1-IRES-GFP* and *Ser1T-IRES-GFP*) into the *BamH1-SalI* site of the *pet23a(+)* vector (Novagen). Finally, a *BamHI* blunted-*SalI* fragment of *Ser1-IRES-GFP* was cloned into the *BamHI* blunted-*NotI* cleaved *RSVpBMN*, *BamHI-NotI* cleaved *Ser1^T-IRES-GFP* was cloned into *BamHI-NotI* site of *RSVpBMN BamHI* partially-digested-*HindIII* fragments of *Dl1-IRES-GFP* and *Dl1^T-IRES-GFP* were cloned into the *BamHI-HindIII* site of the *RSV-Ser1^T-IRES-GFP* viral vector.

Generation of Retrovirus

Dl1, X-Su(H)^{dn} (gift of J.C. Izpisua-Belmonte), and Lfng RCAS retroviral supernatants were generated as described in Henrique *et al.* (1997) and Laufer *et al.* (1997). Pseudotype retroviruses were generated according to Yee *et al.*, (1994). In summary, 80% confluent *293gp* cells (Viron, Qiagen) were co-transfected with the different *RSVpBMN* viral plasmids and a plasmid expressing VSV-G (Witte and Baltimore, 1977; Emi *et al.*, 1991) using Superfect (Qiagen). 24 h post-transfection, the high-serum medium (DMEM, 10% FCS) was replaced by low serum medium (DMEM, 2% FCS), and supernatants were collected 48 h, 72 h and 96 h following transfection, filtered through a 0.45 mm filter (Nalgene), snapfrozen in liquid N_2, and stored at –70°C. Pooled supernatants were concentrated by two steps of ultracentrifugation (25,000rpm/90min) at 4°C (SW28 or SW40 Beckman rotor). The final pellet was homogenised by gentle pipetting, and ~20 µl aliquots snapfrozen in liquid N_2 and stored at –70°C. The viral solution is about 1000-fold more concentrated than the initial supernatants and had a viral titre of about 5.10^8-10^9 cfu/ml.

Embryos and Chicken Injection

Fertile hens's eggs (Light Sussex X Rhode Island Red) were incubated in a humidified atmosphere at 38°C. The Hamburger-Hamilton tables (Hamburger and Hamilton, 1992) were used for staging and to relate stage number to age in notional hours of incubation. About 0.1 µl of virus solution containing 0.8 mg/ml of polybrene (Sigma), 3% methyl cellulose (viscosity 2%, 15 centipoises, Sigma) and fast green (Sigma) was injected in the lumen of the neural tube of stage 10 to stage 12 chicken embryos. The embryos were collected from 18 h to 5 days post-infection. No phenotypes were observed earlier than 24 h post-infection.

In Situ *Hybridisation and Immunohistochemistry*

In situ hybridisation was performed using NBT/BCIP or Fast Red substrates (Myat *et al.*, 1996; Eddison *et al.*, 2000). Immunofluorescence on whole mount spinal cords or 15 µm thick transverse cryostat sections was analysed by confocal microscopy (BioRad MRC600). The sections and whole mount spinal cords were incubated overnight/4°C in blocking solution (PBS solution, 3% BSA, 10% FCS, 0.1% Triton) containing the primary antibody. Ser1 was detected with a rabbit polyclonal antiserum directed against the intracellular domain of the chick Ser1 protein (Adam *et al.*, 1998). This epitope is missing from Ser1^T. Dl1 was detected with a rabbit antiserum directed against the amino-acids 325-462 from the extracellular domain of the chick Dl1 protein (Henrique *et al.*, 1997). GFP was visualised by

immunohistochemistry using a rabbit polyclonal serum (gift from D. Shima, CR-UK). The RCAS infection was monitored using an rabbit polyclonal serum against the gag-p27 viral protein (Potts *et al.*, 1987) The Developmental Studies Hybridoma Bank supplied antibodies against Shh, Islet1/2, Nkx2.2 and HNF3β. Anti-TUJ1 (βIII-tubulin) was provided by Babco. Secondary antibodies were labelled with Peroxydase (Jackson lab.) Alexa-488 or Alexa-594 (Molecular Probes). Only heavily infected embryos were analysed; descriptions are based on at least 3 embryos per condition and on at least 24 sections per antibody. Tunel staining was performed according to the manufacturer's protocol (Promega)

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