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Multiple *delta* genes and lateral inhibition in zebrafish primary neurogenesis

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SUMMARY

In *Drosophila*, cells are thought to be singled out for a neural fate through a competitive mechanism based on lateral inhibition mediated by Delta-Notch signalling. In tetrapod vertebrates, nascent neurons express the *Delta1* gene and thereby deliver lateral inhibition to their neighbours, but it is not clear how these cells are singled out within the neurectoderm in the first place. We have found four *Delta* homologues in the zebrafish – twice as many as reported in any tetrapod vertebrate. Three of these – *deltaA*, *deltaB* and *deltaD* – are involved in primary neurogenesis, while two – *deltaC* and *deltaD* – appear to be involved in somite development. In the neural plate, *deltaA* and *deltaD*, unlike *Delta1* in tetrapods, are expressed in large patches of contiguous cells, within which scattered individuals expressing *deltaB* become singled out as primary neurons. By gene misexpression experiments, we show: (1) that the singling-out of primary neurons, including the unique Mauthner cell on each side of the hindbrain, depends on Delta-Notch-mediated lateral inhibition, (2) that *deltaA*, *deltaB* and *deltaD* all have products that can deliver lateral inhibition and (3) that all three of these genes are themselves subject to negative regulation by lateral inhibition. These properties imply that competitive lateral inhibition, mediated by coordinated activities of *deltaA*, *deltaB* and *deltaD*, is sufficient to explain how primary neurons emerge from proneural clusters of neuroepithelial cells in the zebrafish.

Key words: *delta*, Lateral inhibition, Zebrafish, Neurogenesis, Mauthner neuron, *notch*

INTRODUCTION

Both in vertebrates and in invertebrates, neurons originate as isolated cells within a neurogenic neuroepithelium; in the neurogenic regions, many cells have the potential to follow a neural pathway of differentiation, but only a scattered subset do so at any one time. The Delta-Notch lateral-inhibition mechanism plays a key part in this process, by preventing the immediate neighbours of each nascent neural cell from simultaneously embarking on neural differentiation. In vertebrates, this has been established mainly through study of the effects of misexpressing the *Delta1* gene (Austin et al., 1995; Chitnis et al., 1995; Dorsky et al., 1997; Henrique et al., 1997a). These vertebrate observations pose an unsolved problem, however, as to how the neural cells become singled out from their neighbours in the first place.

This question has been examined in *Drosophila*, with regard to the neuroblasts of the central nervous system and the sensory mother cells of the peripheral nervous system. In both cases, the process appears to occur in two steps (Campos-Ortega, 1993; Ghysen et al., 1993). First, a cluster or patch of cells switches on expression of one or more proneural genes, conferring the potential for a neural fate. Then, within this proneural cluster, a second set of genes, the neurogenic genes, are brought into play to allow only a subset of the cells actually to become committed to neural differentiation.

The neurogenic genes code for components of a cell-cell signalling pathway, in which the transmembrane protein Notch serves as receptor and the transmembrane protein Delta as its ligand (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995). Delta in one cell activates Notch in its neighbour, thereby delivering a lateral inhibitory signal – that is, inhibiting the neighbour from becoming committed to neural differentiation. The neurogenic genes, and in particular *Delta*, are under the control of the proneural genes and, in *Drosophila*, are expressed at the outset throughout the proneural cluster (Haenlin et al., 1990; Kooh et al., 1993; Hinz et al., 1994; Kunisch et al., 1994); thus it appears that all the cells in the cluster initially both deliver inhibition to one another and receive it. To explain how the system advances to a state where one cell – the prospective neural cell – escapes from inhibition, while its neighbours remain inhibited, it has been proposed that the activity of Delta itself is regulated by lateral inhibition, so that the more inhibition a cell receives from its neighbours, the less it is able to deliver back to them (Goriely et al., 1991; Heitzler and Simpson, 1991). This gives rise to a positive feedback loop that will tend to amplify any initial difference between neighbouring cells: it makes lateral inhibition a competitive process, allowing a single

winner to emerge in each small region, delivering inhibition to its neighbours but receiving none back from them (Sternberg, 1993; Chitnis, 1995; Collier et al., 1996; Lewis, 1996).

Evidence to support this feedback model has come from analysis of the pattern of production of neural cells at the borders of mutant clones in the *Drosophila* epidermis (Heitzler and Simpson, 1991). Moreover, in the nematode worm, where a homologous system of genes serves to single out one cell for a special fate in the developing vulva, the process has been shown to involve a lateral inhibition feedback loop in which the *Delta* homologue (*lag-2*) is regulated at the RNA level (Wilkinson et al., 1994). In *Drosophila*, the mode of regulation of *Delta* seems to vary from tissue to tissue (Parks et al., 1995; Heitzler et al., 1996; de Celis and Bray, 1997; Seugnet et al., 1997). There is a defined molecular pathway operating in peripheral neurogenesis through which activation of Notch can inhibit transcription of *Delta* (Heitzler et al., 1996), but direct observations of *Delta* expression in the fly's embryonic central nervous system, by in situ hybridisation or with antibodies, fail to show the expected pattern of regulation in proneural clusters (Kooh et al., 1993), suggesting that the regulation of Delta activity by lateral inhibition is post-translational or dependent on some ancillary factor, or that prospective neural cells in the embryonic CNS are marked out by some prior molecule that makes them resistant to lateral inhibition (Seugnet et al., 1997).

Recent work has shown that birds, amphibians and mammals possess homologues of *Delta* and *Notch*, and that neurogenesis is regulated, as in the fly, by Delta-Notch signalling (Chitnis et al., 1995; Lewis, 1996; Henrique et al., 1997a). There is, however, a contrast with *Drosophila* in one important respect: in the vertebrate examples studied so far, *Delta1* expression has been detected only in the nascent neurons, and not in their neighbours in the neurogenic region (Chitnis et al., 1995; Henrique et al., 1995; Myat et al., 1996). This raises the question whether there is a fundamental difference between *Drosophila* and vertebrates in the way in which the *Delta* genes are regulated and in the mechanism by which neural cells are singled out for their fate.

In this paper, we report the cloning of four *Delta* homologues from the zebrafish, and examine their function in primary neurogenesis – that is, in the formation of the earliest neurons in the CNS. We show that three of the genes, *deltaA*, *deltaB* and *deltaD*, are expressed in the neurogenic regions. But, in contrast with previous studies in vertebrates, their expression there is not restricted to the prospective primary neurons: *deltaA* and *deltaD* are expressed widely, in large groups of contiguous cells at sites of neurogenesis. Within these groups, some cells express the genes at higher levels, and these cells, which express *deltaB* also, can be identified as prospective neurons. By functional tests we show, furthermore, that the fish *delta* genes control primary neurogenesis through lateral inhibition, and that this inhibition operates in each case with feedback based on regulation of *delta* expression at the RNA level, providing a competitive Delta-Notch mechanism to single out the cells that are to differentiate as neurons.

MATERIALS AND METHODS

Fish rearing and embryo culture

Zebrafish eggs were obtained by natural spawnings from a colony of fish derived from stock from 'The Goldfish Bowl' pet shop, Oxford, England. Eggs were collected and maintained at 28.5°C in system water or E3 embryo medium (Haffter et al., 1996) with 10^{-5}% methylene blue to inhibit fungal growth. Embryos were staged according to Kimmel et al. (1995); embryonic ages are given in hours postfertilization (hpf) at 28.5°C.

Cloning and sequencing zebrafish *delta* genes

Zebrafish *Delta/Serrate* homologues were cloned by PCR, as described by Henrique et al. (1995), using similar primers, targeted to the DSL domain and an adjacent EGF repeat. The initial reaction used cDNA made from a 24 hour zebrafish random-primed cDNA library (gift of U. Strähle). Degenerate oligonucleotide primers TTCTGCCGICCICGIGA(C/T)GA(C/T) and TCIATGCAIGTIC-CICC(A/G)TT were used. 40 cycles of amplification yielded products varying in length from approximately 300 to 700 base pairs. Amplified fragments were cloned into pBluescript KS- (Stratagene) and sequenced. Two of the PCR fragments were used to screen a 20-28 hour zebrafish λZAP cDNA library (made by Robert Riggleman and Kathryn Helde, a gift from David Grunwald), yielding clones that corresponded to four distinct *delta* genes. cDNAs were excised from the λZAP II vector using Stratagene's Rapid Excision Kit, and subjected to double-stranded sequencing in both directions using ^{35}S, with the Pharmacia T7 polymerase kit and double-stranded nested-deletion kit. Sequences were analysed using the Wisconsin GCG computer programs.

Whole-mount in situ hybridisation and antibody staining

Digoxigenin- or fluorescein-labelled RNA antisense probes were generated with a Stratagene RNA transcription kit. Enzymes for linearisation and transcription for probe synthesis were as follows: *islet-1* (Inoué et al., 1994) – *Xba*I and T3; *paxb* (Krauss, 1991) – *Bam*HI and T7; *krox20* (Oxtoby and Jowett, 1993) – *Xba*I and T3; and *notch* (Bierkamp and Campos-Ortega, 1993) – *Xba*I and T7. Clones for *deltaA*, *deltaB* and *deltaD* were cut with *Eco*RI and transcribed with T7, while the clone for *deltaC* was cut with *Xba*I and transcribed with T7.

Whole-mount in situ hybridisation followed Oxtoby and Jowett (1993) with minor modifications. Two-colour whole-mount in situ hybridisation was carried out essentially as described by Hauptmann and Gerster (1994).

To show Mauthner cells, embryos at 30 hpf were stained with the 3A10 monoclonal antibody (Furley et al., 1990) as described by Hatta (1992).

Mounting and photography

Specimens were stored in PTW (PBS + 0.1% Tween20) with 0.1% sodium azide at 4°C and were photographed in 100% glycerol, either intact or as flat mounts dissected off the yolk and flattened under a coverslip. Images were taken using a Leitz Diaplan microscope or an MRC-600 confocal microscope (BioRad) and assembled into figures using Adobe Photoshop.

Zebrafish embryo injections

Embryos were injected at the 1- to 4-cell stage, with 200 pl of a solution of 20-50 ng/μl RNA in water, unless otherwise stated (see Tables 2, 3 and 4), containing 0.2% phenol red. After injection, the embryos were allowed to develop until the 5- to 10-somite stage. Embryos injected with *lacZ* mRNA were fixed for 30 minutes at room temperature in 4% paraformaldehyde in PTW. After three washes in PTW, the embryos were stained for β-galactosidase activity by incubation for 30 to 90 minutes at 37°C in 400 μg/ml X-gal, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 4 mM $MgCl_2$, in PTW. The X-gal staining was not allowed to develop for more than 90 minutes in order to limit RNA degradation and embryo damage, and to avoid masking the in situ staining. After X-gal staining, the embryos were fixed overnight at 4°C and then dehydrated in methanol

and processed for whole-mount in situ hybridisation as described above. Embryos not injected with *lacZ* mRNA were fixed overnight at 4°C in 4% paraformaldehyde in PBS and then dehydrated in methanol and processed for whole-mount in situ hybridisation.

To make sense RNA for injections, *X-Delta1*, *X-Delta1*^{dn} and *X-NotchΔE* constructs (Coffman et al., 1993; Chitnis et al., 1995) were linearized by *Not*I and transcribed with SP6 RNA polymerase. A plasmid corresponding to an *Xba*I-*Not*I fragment of the NLSlacZ sequence, containing the whole *lacZ* open reading frame with a nuclear localisation signal (a gift of Jonathan Pearce), inserted into the CS2 vector (Turner and Weintraub, 1994), was linearized with *Acc*65I and transcribed with SP6 RNA polymerase. The *deltaB* transcription construct was made by replacing the *Nsi*I-*Xho*I fragment containing part of the 3′ UTR of *deltaB* in Bluescript by a *Nsi*I-*Sma*I fragment containing beta-globin 3′ sequences and poly(A) sequences from pSP64T (Krieg and Melton, 1984). The construct was linearized by *Acc*I and transcribed with T3. In vitro preparation of capped mRNAs was performed with the MegascriptTM kit (Ambion).

RESULTS

The zebrafish has at least four genes homologous to *Delta*

By screening a zebrafish 24-hour cDNA library by PCR with primers based on the *Drosophila Delta* gene (see Materials and Methods), we identified fragments of four distinct zebrafish *Delta* homologues. We called these *deltaA, deltaB, deltaC* and *deltaD*. An exchange of sequence data revealed that our *deltaA* and *deltaD* were identical with genes independently discovered by B. Appel and J. Eisen in Oregon (Appel and Eisen, 1998) and also (in the case of *deltaD*) by P. Dornseifer, C. Takke, and J. Campos-Ortega in Köln (Dornseifer et al., 1997). While we have determined the complete cDNA sequence of *deltaB* (GenBank accession number AF006488), they have determined that of *deltaA* and *deltaD*, and have kindly allowed us to include their sequence data in our Fig. 1, for comparison with *deltaB*. The full sequence of *deltaC* will be presented elsewhere; as discussed below, it is not expressed in the neural plate during primary neurogenesis.

The deduced sequences of the proteins DeltaA (765

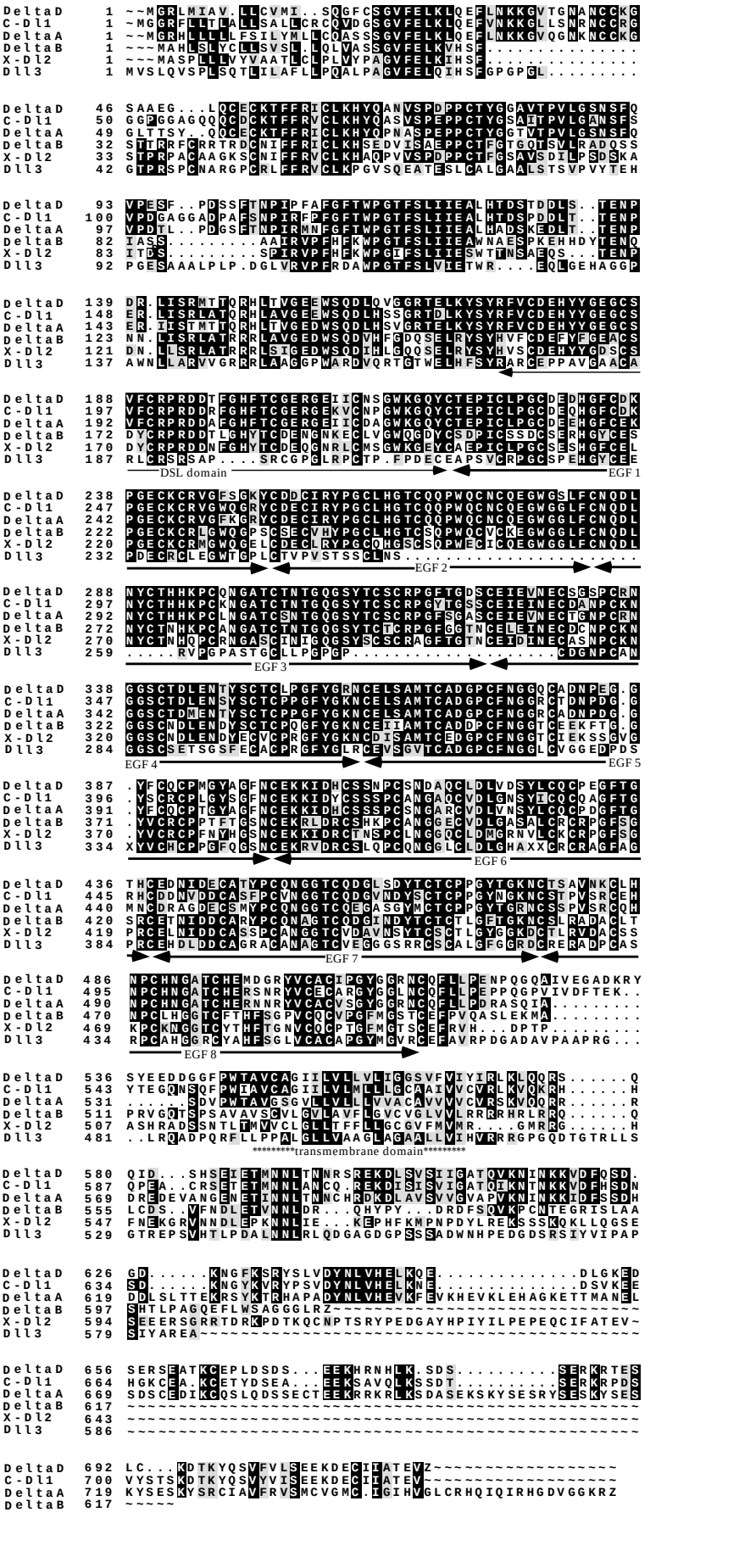

Fig. 1. Amino acid sequences of the zebrafish DeltaA, DeltaB and DeltaD proteins, deduced from the cDNA sequences, aligned with chick Delta1 (C-Dl1), *Xenopus* Delta2 (X-Dl2) and mouse Dll3. The highly conserved DSL domain, the eight EGF repeats and the transmembrane (TM) domain are indicated. Note that DeltaA has five contiguous repeats of a tyrosine-rich motif *S*YS (mainly ESKYS) near its intracellular terminus, representing a possible tyrosine phosphorylation site. The DeltaA sequence shown assumes that translation begins at the methionine indicated, and not at another methionine codon that lies 114 nucleotides upstream and would correspond to a protein 38 amino acids longer; the nucleotide sequence at the former methionine matches a vertebrate consensus translation initiation sequence, whereas that at the latter does not (see Appel and Eisen, 1998). (*deltaB*, GenBank accession number, AF006488.)

amino acids), DeltaB (616 amino acids) and DeltaD (718 amino acids) all have a size and domain structure similar to that of the *Drosophila* Delta protein: there is a large (>500 amino acids) N-terminal region corresponding to the extracellular portion of Delta, a short hydrophobic region corresponding to the transmembrane segment, and a C-terminal region corresponding to the intracellular portion of Delta. Sequence conservation is clear throughout the extracellular region (46-47% amino-acid identity with Delta for all three fish genes); this region includes a typical strongly conserved DSL (Delta-Serrate-Lag2 homology) domain (Tax et al., 1994) and 8 EGF repeats (as compared with 9 for *Drosophila* Delta). The intracellular domain, however, shows a striking lack of conservation (≤17% amino-acid identity with *Drosophila* Delta). The contrast between extracellular and intracellular domains in their degree of conservation is also evident when we compare the fish Delta proteins with one another and with the chick C-Delta1 protein (Henrique et al., 1995). Thus DeltaA and DeltaD, which are most similar to one another, show 80% identity in their extracellular regions but only 47% identity in their intracellular regions; they both show 77% identity to C-Delta1 extracellularly, but, respectively, 47% and 67% identity to it intracellularly.

DeltaB is more divergent: in all three regions – extracellular, transmembrane and intracellular – it differs from DeltaA and DeltaD by roughly the same amount as it does from C-Delta1, and more than DeltaA or DeltaD does from C-Delta1. This suggests that it may have arisen through a gene duplication/divergence event predating the divergence of fish from tetrapods. Its extracellular domain is 54-57% identical to that of DeltaA, DeltaD and C-Delta1, while its intracellular domain (75 amino acids) is much shorter than theirs and shows only 18-27% identity. As shown in Table 1, DeltaB bears a slightly closer resemblance to the Xenopus Delta2 protein (Jen et al., 1997) (59% identity overall) than to Delta1 (52%). All three fish *delta* genes are as much diverged from mouse *Delta-like-3 (Dll3)* (Dunwoodie et al., 1997) as they are from *Drosophila Delta*.

The high degree of extracellular sequence conservation among the members of the Delta family reflects the known importance of this region for binding to Notch and possibly other proteins (Lieber et al., 1992); the variability of the intracellular region, evident in virtually all pairwise comparisons of one Delta with another, suggests that this part of the molecule has a less critical function, or one that is less dependent on specific binding to other proteins.

deltaC and *deltaD* are expressed in presomitic mesoderm and in recently-formed somites

Expression of all four genes – *deltaA*, *deltaB*, *deltaC* and *deltaD* – begins to be visible by in situ hybridisation during epiboly (Fig. 2A-D). *deltaC* and *deltaD* are both strongly expressed in the germ ring, throughout the region of involuting mesoderm, with the exception of the axial (midline) region – the region of the prospective notochord. Paired bilateral transverse stripes of expression, first of *deltaC* and then of *deltaD*, are seen a little more anteriorly, foreshadowing the formation of somites. As epiboly comes to an end, the mesodermal expression of *deltaC* and *deltaD* resolves into a strong tail-bud domain and, more anteriorly, bilateral pairs of stripes probably corresponding to somites that are about to form. Low-level expression persists subsequently in recently formed somites as well as in the presomitic mesoderm, at least up to 24 hpf (Figs 3-5): *deltaC* is expressed in the posterior parts of somites, *deltaD* in the anterior parts (Fig. 5). In their mesodermal expression in relation to somite development, *deltaC* and *deltaD* thus resemble the *Delta1* gene of mouse, chick and frog (Bettenhausen et al., 1995; Chitnis et al., 1995; Henrique et al., 1995; Dornseifer et al., 1997; Hrabé de Angelis et al., 1997), the *Delta2* gene of *Xenopus* (Jen et al., 1997), and the *Dll3* gene of the mouse (Dunwoodie et al., 1997). The fish *deltaA* and *deltaB* genes, by contrast, do not appear to be expressed in the developing mesoderm at these early stages. The development of the mesoderm and its dependence on *deltaD* are discussed by Dornseifer et al. (1997) and will not be considered further here.

The expression patterns of *deltaA*, *deltaB* and *deltaD* foreshadow primary neurogenesis

Our main concern here is with the developing nervous system. By 90% epiboly (9 hpf), transcripts of *deltaA*, *deltaB* and *deltaD* are seen in the epiblast (the future neurectoderm), in or near the axial midline and in two anterolateral pairs of patches (Fig. 2A,B,D). The patterns for the three genes appear similar

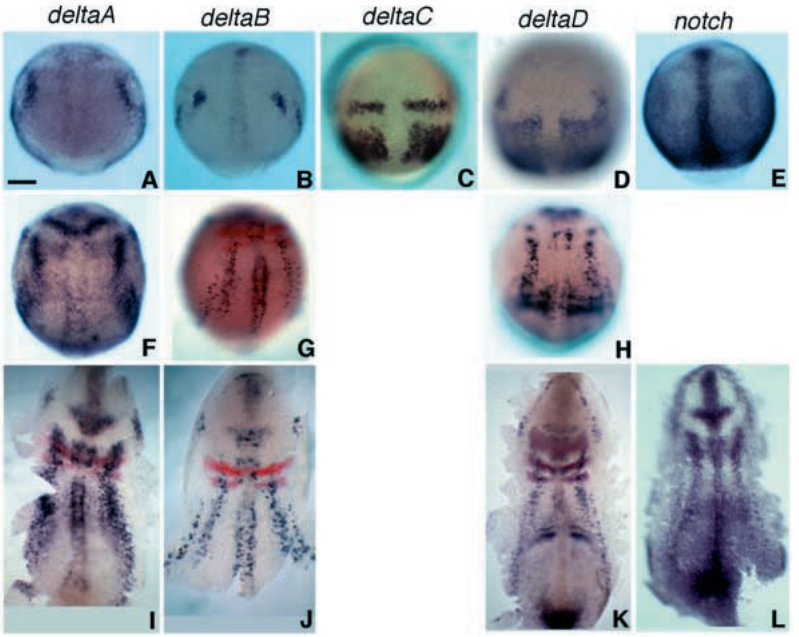

Fig. 2. Early expression patterns of all four zebrafish *delta* genes and of *notch* seen by in situ hybridisation. Dorsal views, anterior to the top. (A-E) At 90% epiboly (9 hpf); (F-L) at bud to 1-somite stage (10-10.5 hpf). The middle row (F-H) shows intact embryos, the bottom row (I-L), dissected flat mounts. Red stain in G and in I-K shows expression of *krox20* and, in K, of *paxb*. Tissue sections (not shown) confirm that *deltaA* and *deltaB* are expressed in the epiblast, in prospective neural tissue, whereas the strong expression of *deltaC* and *deltaD* at these stages is in the prospective mesoderm. Scale bar: 100 μm.

Table 1. Comparisons of zebrafish, chick, *Xenopus* and *Drosophila* Delta proteins

	Zebrafish proteins		
	DeltaA	DeltaB	DeltaD
Full length, aa	765	616	718
Intracellular domain, aa	208	75	150
Extracellular domain, aa	536	519	547
No. of EGF repeats	8	8	8
Full length comparisons			
Zebrafish DeltaA	(100%)	52%	72%
Zebrafish DeltaB	49%	(100%)	50%
Zebrafish DeltaD	72%	50%	(100%)
Chick Delta1	69%	52%	74%
Drosophila Delta	36%	41%	39%
Xenopus Delta2	48%	59%	48%
Mouse Dll3	33%	42%	34%
Intracellular domain comparisons			
Zebrafish DeltaA	(100%)	23%	47%
Zebrafish DeltaB	23%	(100%)	27%
Zebrafish DeltaD	47%	27%	(100%)
Chick Delta1	47%	18%	67%
Drosophila Delta	14%	15%	17%
Xenopus Delta2	21%	23%	21%
Mouse Dll3	17%	20%	21%
Extracellular domain comparisons			
Zebrafish DeltaA	(100%)	54%	80%
Zebrafish DeltaB	54%	(100%)	56%
Zebrafish DeltaD	80%	56%	(100%)
Chick Delta1	77%	57%	77%
Drosophila Delta	47%	46%	47%
Xenopus Delta2	55%	65%	56%
Mouse Dll3	35%	46%	34%

Percentages represent percent amino-acid identity, computed by the Wisconsin GCG 'Gap' program.

but not identical (although the rapidity of change during epiboly makes it difficult to make precise comparisons between separately labelled specimens): for example, *deltaB* expression is seen in the midline while *deltaA* is expressed just lateral to this. Comparison with later stages suggests that the anterolateral patches correspond to the future sites of origin of the primary sensory neurons of the trigeminal ganglion and of the posterior CNS (the Rohon-Beard cells), and that the medial bands of *deltaA* expression flanking the midline correspond to the domains within which primary motor neurons will arise. Meanwhile, we detect no expression of *deltaC* in the prospective neural regions. Expression of the zebrafish *notch* gene (Bierkamp and Campos-Ortega, 1993), corresponding to tetrapod *Notch1*, is ubiquitous, but strongest in the dorsal midline region (Fig. 2E).

By the 1-somite stage (10.3 hpf) (Fig. 2F-L), the neural plate is well defined and paraxial stripes of expression of *deltaA*, *deltaB* and *deltaD* are seen, marking sites of primary neurogenesis. At the 5-somite stage (11.7 hpf), the patterns of gene expression are similar but more sharply delineated; neurogenesis has begun, as indicated by expression of the neuronal marker *islet1* (Korzh et al., 1993; Inoué et al., 1994). We have chosen this stage (Fig. 3) for detailed analysis. We used the expression domains of *paxb* (at the midbrain-hindbrain junction) and of *krox20* (in rhombomeres 3 and 5) as landmarks, shown in red by a double in situ hybridisation protocol. A small set of scattered cells expressing *islet1* just lateral to the *paxb* domain could be identified as nascent placode-derived neurons of the trigeminal ganglion. The bands of *islet1*-expressing cells flanking the midline posterior to the *krox20* domains could be identified as nascent primary motor neurons, while those lying laterally, at the margins of the posterior neural plate, could be identified as nascent Rohon-Beard cells. At all these sites, *deltaA*, *deltaB* and *deltaD* are also expressed, and in larger numbers of cells than express *islet1*. Taking these patterns in conjunction with those seen earlier and later, we conclude that expression of *deltaA*, *deltaB* and *deltaD* foreshadows the onset of neuronal differentiation as indicated by *islet1*. The three *delta* genes are also expressed in certain regions of the neural plate where *islet1* expression is absent; these correspond to sites of differentiation of primary interneurons that do not express *islet1*, such as the Mauthner cells, other hindbrain reticulospinal cells and the primary interneurons of the spinal cord, lying in rows just medial to the

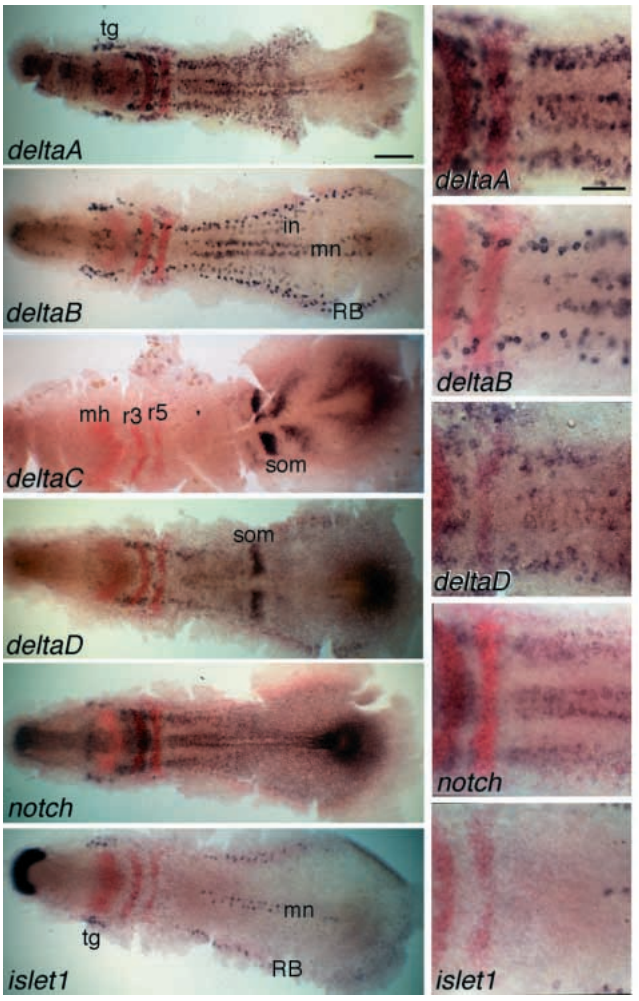

Fig. 3. Expression of *deltaA, B, C* and *D, notch* and *islet1* at the 5-somite stage (11.7 hpf), shown by in situ hybridisation with a purple-blue (NBT/BCIP) detection system; embryos have also been double-labelled with probes against *paxb* and *Krox20*, using a Fast-Red detection system, to provide landmarks. On the right, enlargements of the posterior hindbrain and anterior spinal-cord region are shown. Embryos are flat-mounted; anterior is to the left. tg, trigeminal ganglion; mh, midbrain/hindbrain boundary; r3, r5, rhombomeres 3 and 5; mn, motor neurons; in, interneurons; RB, Rohon-Beard (sensory) neurons; som, prospective somite. Scale bars: 200 μm (whole embryos), 100 μm (details).

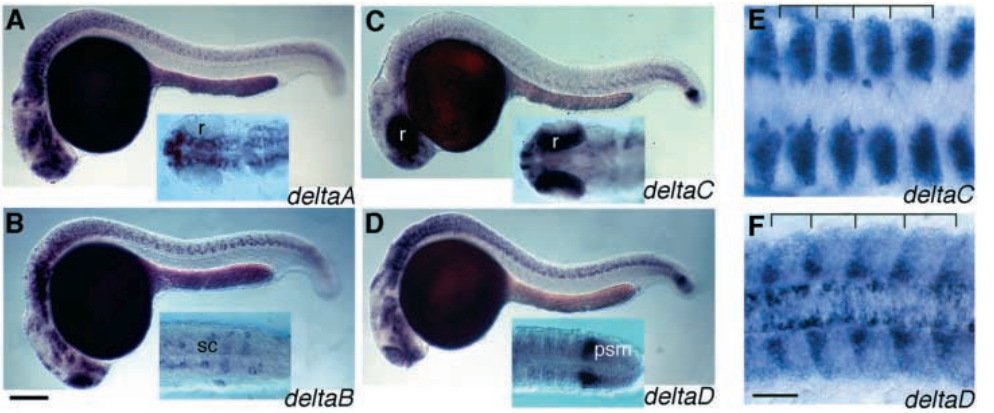

Fig. 4. (A-D) Expression of *deltaA*, *deltaB*, *deltaC* and *deltaD* in whole mounts at 24 hpf. Note expression of *deltaC* and *deltaD* in the presomitic mesoderm (psm) of the tail bud and in recently formed somites. *deltaC* is now also strongly expressed in the retina (r), while *deltaA*, *deltaB*, and *deltaD* are expressed in scattered subsets of cells in the brain and spinal cord (sc). (E,F) Expression of *deltaC* and *deltaD* in recently formed somites at the 10-somite stage (14 hpf). Trunk region of flat-mounted embryo, anterior to the left. Note that *deltaC* is expressed in the anterior part of each somite, *deltaD* in the posterior part. Scale bars: 200 μm (A-D), 50 μm (E,F).

Rohon-Beard cells (Figs 3, 5) (Mendelson, 1986). There is still no noticeable expression of *deltaC* in the neural plate: it will begin to be expressed strongly during secondary neurogenesis, especially in the developing retina (Fig. 4C). *notch* expression, on the other hand, is still widespread, although more intense in regions of *delta* gene expression (see also Bierkamp and Campos-Ortega, 1993).

deltaA and *deltaD* are expressed diffusely, in proneural patches; within these patches, prospective neurons express *deltaA*, *deltaB* and *deltaD* strongly

We have used the 5-somite stage to examine whether the fish *delta* genes are expressed in nascent neurons only, like *Delta1* in tetrapods, or more diffusely, in proneural clusters, like *Delta* in *Drosophila*. Figs 3 (right-hand panels) and 5 show detailed comparative views of the expression of *deltaA*, *deltaB*, *deltaD* and *islet1* probes. While the domains of expression are broadly similar, there are marked differences at the level of the individual cells. *islet1* and *deltaB* are expressed strongly and selectively in scattered cells that are mostly isolated from one another by non-expressing cells (Fig. 5E-H,J). *deltaA* and *deltaD*, meanwhile, are expressed more diffusely, in patches comprising many contiguous cells; in some of these cells the expression is strong, in others weak (Fig. 5I,K). The cells expressing *deltaA* most strongly appear scattered, while those expressing *deltaD* strongly occur more as clusters, but both are concentrated in the neurogenic regions where cells expressing *deltaB* are found.

Double labelling reveals that the cells within a *deltaA* patch that express *deltaA* strongly are precisely the cells that express *deltaB* (Fig. 5A-D); these double-labelled

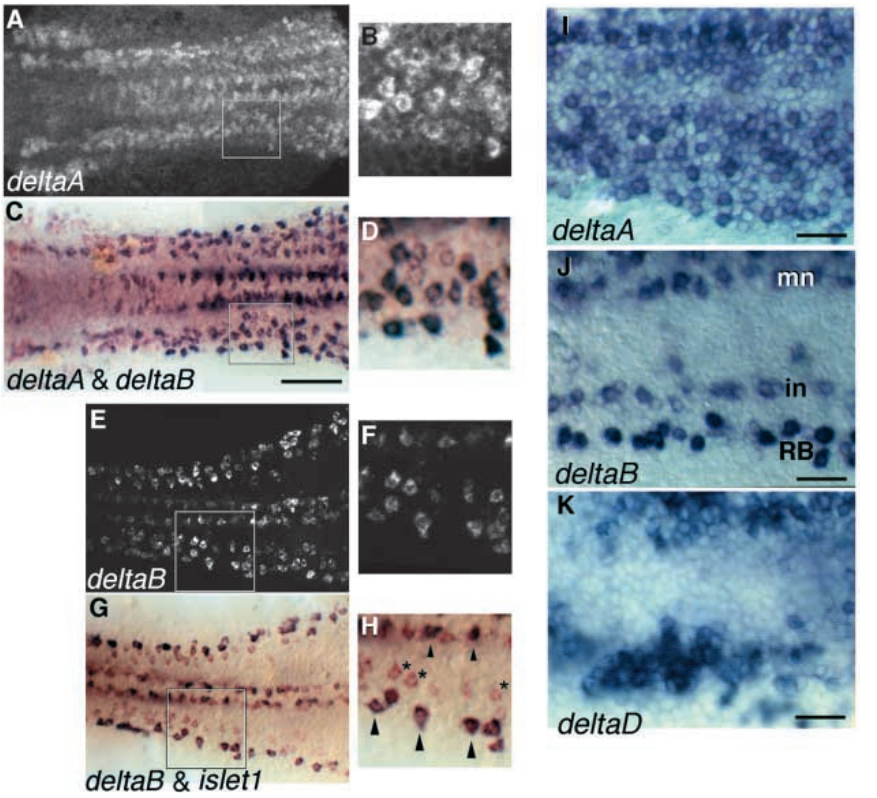

Fig. 5. (A-D) Coexpression of *deltaA* and *deltaB* in the same cells in the neural plate at the 5-somite stage. The boxed regions on the left are shown enlarged on the right (B,D). The cells expressing *deltaB* are generally the same that express *deltaA* most strongly. The embryo was first stained for *deltaA* expression, revealed by in situ hybridisation using fluorescent Fast Red detection and was viewed intact by epifluorescence, using the confocal microscope to construct an extended-focus image. The same specimen was then hybridised with a probe for *deltaB*, revealed in purple with NBT/BCIP, and was flat-mounted and photographed with bright-field optics. Sequential imaging was used because the dark NBT/BCIP stain often obscures the Fast Red fluorescence. (E-H) Coexpression of *deltaB* and *islet1* in the neural plate at 5 somites, shown by double in situ hybridisation. *deltaB* in red (fluorescence in upper panel (E,F), bright field in lower panel (G,H)), *islet1* in blue-black (bright field, lower panel). Primary motor neurons and Rohon-Beard cells express both genes (arrowheads); cells expressing *deltaB* but not *islet1* are probably primary interneurons (asterisks). (I-K) Details of the prospective anterior spinal region at the 5-somite stage, showing the diffuse but uneven expression of *deltaA* and *deltaD* in large patches of contiguous cells, and the more restricted expression of *deltaB* in scattered, isolated cells. In each case, the left side of the neural plate is shown, with midline at the top and anterior to the left of the picture. Scale bars: 100 μm (A-H), 50 μm (I-K).

cells lie next to the midline (the site of origin of primary motor neurons), or at or just medial to the lateral edges of the *deltaA* domain (the sites of origin of Rohon-Beard cells and primary interneurons, respectively). Almost all (92%; 713 cells out of 777 counted, 6 embryos) of the *deltaB*-expressing cells express *deltaA*. Double labelling with *deltaB* and *islet1* (Fig. 5E-H) shows, furthermore, that a large proportion (46%; 233 cells out of 503 counted, 7 embryos) of the *deltaB*-expressing cells also express *islet1*, while almost all (84%; 233 cells out of 278 counted, 7 embryos) of the *islet1*-expressing cells also express *deltaB*. The cells that express *deltaB* but not *islet1* lie just medial to the presumptive Rohon-Beard cells and, from their location and time of appearance, are presumably primary interneurons. We conclude, in short, that cells expressing *deltaB* are nascent neurons, and that the nascent neurons are a subset of the cells expressing *deltaA* and *deltaD*.

Delta-Notch signalling controls the production of primary neurons in the zebrafish

To test whether signalling by Delta protein does indeed regulate commitment to a neural fate in the fish, we injected mRNA coding for different forms of the Delta protein into zebrafish embryos at the 1- to 4-cell stages, and observed the distribution of the *islet1*-positive cells in these embryos at the 5- to 10-somite stages. In some experiments, *E. coli lacZ* mRNA was coinjected as a marker for the presence of injected RNA, its protein product being detected by X-gal histochemistry. In most of the injected embryos, the X-gal staining was unevenly distributed and, in some cases, it was unilateral, which allowed comparison of the *islet1* staining on the affected (sky blue) and control (white) sides.

To overactivate the Delta-Notch signalling pathway, we first injected mRNA coding for the full-length *Xenopus* Delta1 protein (Chitnis et al., 1995). This caused a reduction in the numbers of *islet1*-positive presumptive neurons of all classes (Fig. 6A,B). A similar result was obtained after injection of an mRNA coding for the full-length zebrafish DeltaB protein (Fig. 6E,F; Table 2). Similar experiments by Appel and Eisen (1998) and by Dornseifer, Takke and Campos-Ortega (Dornseifer et al., 1997) have likewise demonstrated inhibition of neurogenesis following injection of mRNA coding for DeltaA and DeltaD. The effects on neurogenesis seen with *deltaB* RNA were, however, much weaker than those with *X-Delta1* RNA and were accompanied, at high doses, by distortions of the neural plate suggestive of disturbances occurring during gastrulation (see Tables 2 and 3).

For comparison with the effects of the Delta proteins, we injected RNA coding for NotchΔE, an extracellularly truncated form of X-Notch1 that is constitutively active and has been found in analogous experiments in *Xenopus* both to inhibit neurogenesis and to cause distortions of the neural plate (Coffman et al., 1993; Chitnis et al., 1995). The effects of injecting *NotchΔE* RNA in the fish were similar, resembling those of *deltaB* RNA (Table 3). The relatively weak inhibition of neurogenesis seen in both cases may reflect relatively short half-lives of these RNAs.

To test the effect of blocking the Delta-Notch signalling pathway, we injected RNA coding for the truncated Delta protein X-Delta1^{dn} (formerly known as X-Delta1^{STU}), which lacks most of its intracellular domain and is known in other species to have a dominant-negative action (Chitnis et al., 1995; Sun and Artavanis-Tsakonas, 1996): it acts by making the cells that express it insensitive to lateral inhibition (Henrique et al., 1997a). The result was a striking increase in

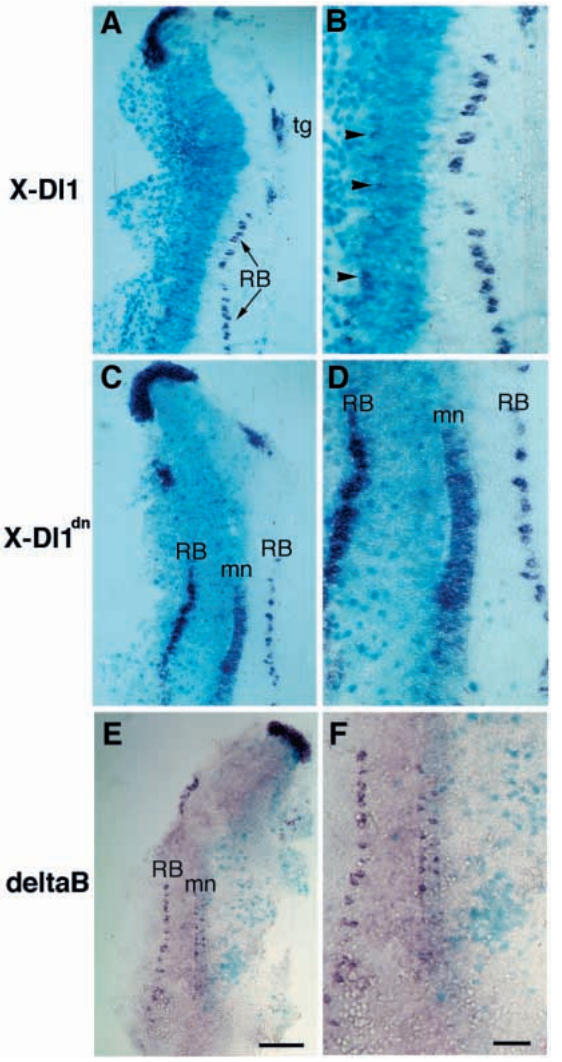

Fig. 6. (A-D) Effects on primary neurogenesis following injection of *X-Delta1* or *X-Delta1^{dn}* RNA, together with *lacZ* RNA as a marker, into one blastomere. Flat-mounted embryos at 5- to 6-somite stage, with *islet1* expression in purple-blue, *lacZ* marker in sky-blue; low-magnification views on the left, details on the right; note that the X-gal treatment of the embryos for detection of lacZ product results in fainter in situ hybridisation stainings than in Figs 2-5. X-Delta1 inhibits production of all classes of *islet1*-positive primary neurons in the injected (sky-blue) region; X-Delta1^{dn} does the opposite. Arrowheads in B indicate a few Rohon-Beard cells that have been formed despite the injected *X-Delta1*. Note that convergence movements and folding of the neural plate lead to cell mixing in the midline, so that motor neuron production appears affected uniformly on both sides of the midline. tg, trigeminal ganglion; mn, motor neurons; RB, Rohon-Beard neurons. (E,F) Effects on primary neurogenesis following injection of *deltaB* RNA, together with *lacZ* RNA as a marker, into one blastomere. Flat-mounted embryos at 5- to 6-somite stage, with *islet1* expression in purple-blue, *lacZ* marker in sky-blue; low magnification views on the left, details on the right. Where *deltaB* RNA is present, production of *islet1*-positive primary neurons is inhibited. Scale bars: 200 μm (A,C,E), 100 μm (B,D,F).

Table 2. Effect on primary neurogenesis of different forms of *Delta* mRNA coinjected with *lacZ* mRNA, as assayed by *islet1* in situ hybridisation + X-gal staining

	mRNA injected				
	X-Delta1 + *lacZ*	*deltaB* (5 ng/μl) + *lacZ*	*deltaB* (40 ng/μl)* + *lacZ*	*X-Delta1^{dn}* + *lacZ*	*lacZ*
Total number of embryos with X-gal staining	21	40	70	13	15
Embryos with less *islet1*-positive cells in the X-gal-stained region	19	10	62	0	0
Embryos with more *islet1*-positive cells in the X-gal-stained region	0	0	0	13	0
Embryos showing no effect	2	30	8	0	15

*In this series of *deltaB* injections, about 50% of the embryos showed marked abnormalities, including widening and shortening of the embryo or localization of the polster cells under the neural plate, suggesting abnormal cell movements during gastrulation (see also Table 3). These abnormal embryos were not scored for the phenotype.

Table 3. Comparison of effects of *Notch* and *Delta* mRNA constructs, injected alone and in combination, as assayed by *islet1* in situ hybridisation

	mRNA injected					
	NotchΔE (10 ng/μl)	*NotchΔE* (1 ng/μl)	*deltaB* (20 ng/μl)	*X-Delta1* (20 ng/μl)	*X-Delta1^{dn}* (20ng/μl)	*X-Delta1^{dn}* (20 ng/μl) + *NotchΔE* (10 ng/μl)
Total number of embryos	48	14	78	26	43	38
Embryos with less *islet1*-positive neurons	10	1	9	16	0	4
Embryos with more (and clustered) *islet1*-positive neurons	0	0	0	0	40	10
Embryos showing no effect on number of *islet1*-positive neurons	15	12	65	0	0	13
Not interpretable (serious gastrulation defects)	23	1	13	10	3	11

the number of *islet1*-positive cells (Fig. 6C,D; Tables 2, 3). Whereas on the control side, these occurred in isolation or in groups of two or three, in corresponding regions on the affected side they occurred in densely packed patches. The powerful effect of *X-Delta1^{dn}* RNA injection was greatly reduced by coinjecting *NotchΔE* RNA (Table 3), as expected if X-Delta1^{dn} acts by preventing activation of endogenous Notch.

Note that injection of RNA coding for a molecule that blocks lateral inhibition (X-Delta1^{dn}) does not have, and would not in general be predicted to have, effects that are the precise inverse of those of RNA coding for an activating molecule (X-Delta1, DeltaB or X-NotchΔE). There are two reasons. First, the RNAs may have different lifetimes. Second, the effects of the blocking molecule are predicted to be irreversible and hence more striking, because cells that prematurely escape inhibition become irreversibly committed to neuronal differentiation; whereas the effects of the activating molecule are reversible and temporary – once the RNA and protein are degraded or diluted away, neuroepithelial cells that were inhibited can differentiate belatedly, so that the final outcome appears more nearly normal.

The above data, taken as a whole, match the results obtained from analogous experiments in *Xenopus* embryos (Chitnis et al., 1995), and likewise indicate that Delta-Notch signalling mediates lateral inhibition in primary neurogenesis.

Delta-Notch signalling controls production of the single Mauthner cell on each side of the brain

In fish and amphibians, a unique giant neuron, the Mauthner cell, is normally generated on each side of the hindbrain, in rhombomere 4. The Mauthner cell is the first to be born of all the neurons in the body (at 7.5 hpf – Mendelson, 1986), and its genesis poses the problem of singling-out in the purest form: what mechanism guarantees that on each side of the brain precisely one cell, and no more, will be assigned this special fate? Analogies with *Drosophila* would suggest that positional signals might first define a small proneural cluster and that lateral inhibition, mediated by Delta-Notch signalling, might

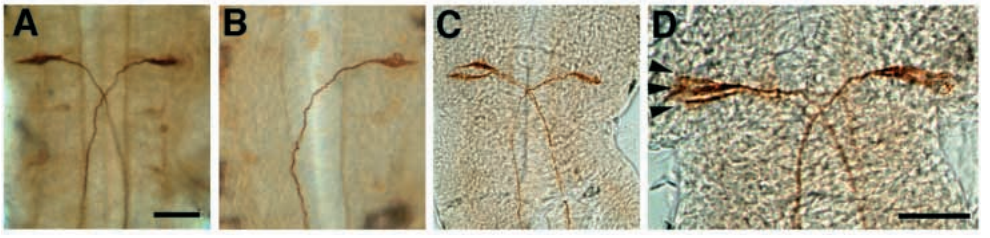

Fig. 7. Effects on Mauthner cell production following injection of *X-Delta1* RNA or *X-Delta1^{dn}* RNA into one blastomere. Embryos were fixed at 30 hpf and stained with 3A10 antibody. (A) Normal control; (B) *X-Delta1* injection, leading to loss of the Mauthner cell on one side of the brain; (C-D) *X-Delta1^{dn}* injections, giving supernumerary Mauthner cells (arrowheads). Scale bars: 100 μm (A-C), 50 μm (D).

then operate within this group of potential Mauthner cells to pick out one of them. If so, forced overactivation of the Delta-Notch pathway should prevent Mauthner cell development, while artificial blockade of the pathway should enable a cluster of cells, instead of one solitary individual, to develop as Mauthner cells. We tested these predictions by injecting either *X-Delta1* or *X-Delta1*^{dn} RNA into an early blastomere and staining with the 3A10 antibody to reveal Mauthner cells at 30 hpf (Fig. 7). Out of 22 embryos injected with *X-Delta1* RNA, 6 had no Mauthner cells, 11 had only one and 5 still had two (one on each side). Conversely, out of 78 embryos injected with *X-Delta1*^{dn} RNA, at least 24 had more than two Mauthner cells, while 54 appeared to have just the usual two. Additional Mauthner cells when present often lie clustered very close together and are not easy to distinguish, so that these figures for the proportion of cases with additional Mauthner cells may be an underestimate.

Delta-Notch signalling regulates expression of *deltaA*, *deltaB* and *deltaD*

The foregoing experiments indicate that lateral inhibition mediated by Delta-Notch signalling is required to prevent neighbours of nascent primary neurons from developing as primary neurons. But is this mechanism also responsible for singling out the primary neurons in the first place, through a competitive process of the type outlined in the Introduction? If so, we should expect a feedback regulation of Delta activity by Delta activity: activation of the Delta-Notch signalling pathway in a given cell should inhibit the production of Delta activity in that cell.

To test this, we analysed the expression of the *deltaA*, *deltaB* and *deltaD* genes in embryos injected with *X-Delta1* or *X-Delta1*^{dn} RNA together with a *lacZ* marker. After injection of *X-Delta1*^{dn} mRNA, the *deltaA* (Fig. 8A), *deltaB* (Fig. 8B) and *deltaD* (Fig. 8C) stainings were both stronger and more extensive on the injected side of the embryo, as compared to the control side. Conversely, after injection of *X-Delta1* mRNA, the *deltaA* (Fig. 8D), *deltaB* (Fig. 8E) and *deltaD* (Fig. 8F) stainings were reduced on the injected side. These results, summarised in Table 4, show that activation of the Notch signalling pathway by a Delta protein represses transcription of *delta* genes and that a blockade of Notch activation permits *delta* genes to be expressed at higher levels and in a larger number of cells than normal. We infer that *deltaA*, *deltaB* and *deltaD* do indeed participate in a feedback loop of the type hypothesised.

In embryos injected with *X-Delta1*^{dn} RNA (Fig. 8A-C), the overexpression of *delta* genes extends over a region significantly broader than the immediate neighbourhood of the normal set of primary neurons: it is not only the cells that would normally be in direct contact with the prospective primary neurons that show signs of having been released from an inhibition. The normal low-level expression of *delta* genes in cells other than the prospective primary neurons is therefore physiologically significant: where its effects are blocked, cells are enabled to display neuronal characteristics inappropriately.

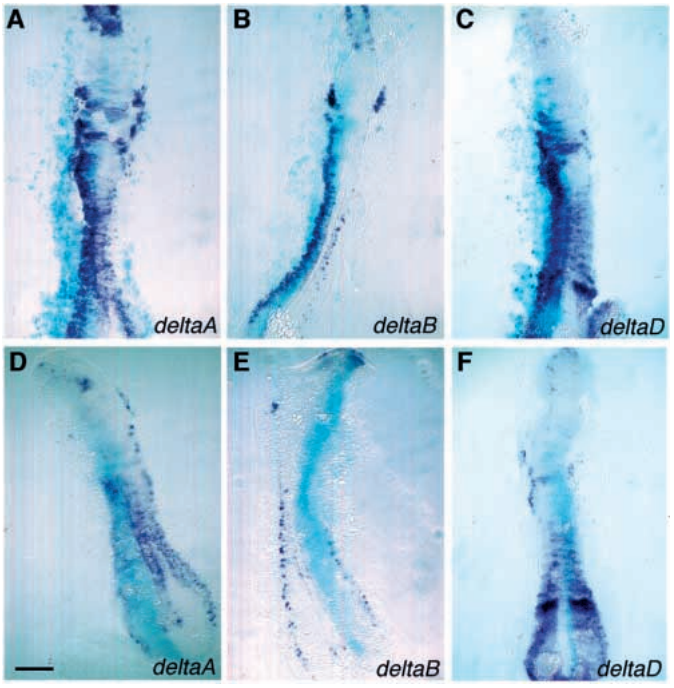

Fig. 8. Effects on expression of *delta* genes in the neural plate following injection of *X-Delta1* or *X-Delta1*^{dn} RNA, together with *lacZ* RNA as a marker, into one blastomere. Flat mounts at 5- to 6-somite stage, with expression of *deltaA*, *deltaB* and *deltaD* in purple-blue, *lacZ* marker in sky-blue. Where *X-Delta1* RNA is present, expression of all three *delta* genes is inhibited; where *X-Delta1*^{dn} RNA is present, they are all overexpressed. Note in A-C that dramatic effects of *X-Delta1*^{dn} are seen even in regions where there is normally only low-level expression of endogenous *delta* genes. Scale bar, 200 μm.

DISCUSSION

One *Delta* gene has so far been described in the chick (Henrique et al., 1995), two in the mouse (Bettenhausen et al., 1995; Dunwoodie et al., 1997) and two in *Xenopus* (Chitnis et al., 1995; Jen et al., 1997). In the zebrafish, we have discovered four. Of these four fish genes, the closest counterpart of the tetrapod *Delta1* gene is *deltaD*: it has the most similar sequence, and, like *Delta1*, is involved both in primary neurogenesis and in somite formation (Dornseifer et al., 1997; Hrabé de Angelis et al., 1997). *deltaC*, by contrast, is expressed in the developing somites but not during primary neurogenesis; in its expression pattern, at least, it resembles the *Xenopus X-Delta2* gene (Jen et al., 1997). *deltaA* and *deltaB* are expressed in regions of primary neurogenesis, like *deltaD*, but not in the developing somites; they do not have precise counterparts among the tetrapod *Delta* genes described so far.

Nascent neurons, expressing *deltaB*, are nested within expression domains of *deltaA* and *deltaD*

We have focused on the roles of *deltaA*, *deltaB* and *deltaD* in primary neurogenesis. We have shown that they have overlapping but significantly different expression patterns in the neural plate: *deltaA* and *deltaD* are expressed in patches of contiguous cells, while *deltaB* is apparently confined to the scattered cells within those patches that differentiate as neurons.

It is tempting to suggest that the *deltaA/deltaD* expression pattern comes first, and that the *deltaB* pattern emerges later,

Table 4. Effect of different forms of *Delta* mRNA, coinjected with *lacZ* mRNA, on the expression of zebrafish *delta* genes

mRNA injected	*X-Delta1 + lacZ*			*X-Delta1^{dn} + lacZ*		
delta gene expression analysed	*deltaA*	*deltaB*	*deltaD*	*deltaA*	*deltaB*	*deltaD*
Total number of embryos with X-gal staining	20	10	29	61	18	24
Embryos with less *delta*-positive cells in the X-gal-stained region	9	6	25	0	0	0
Embryos with more *delta*-positive cells in the X-gal-stained region	0	0	0	53	15	22
Embryos showing no effect	1	0	0	0	0	0
Not scorable (no X-gal staining, or embryo grossly abnormal)	10	4	4	8	3	2

In *lacZ* control injections, there was never a specific effect (reduction or augmentation) on the *deltaA*, *deltaB* or *deltaD* stainings on the lacZ-positive side, although there was sometimes an overall reduction of the in situ staining as compared to control uninjected embryos, probably due to the X-gal staining procedure.

but we have not been able to resolve the temporal sequence: the first signs of expression of all three genes are first seen at a similar time, during epiboly; and at subsequent stages (see Fig. 2), as landmarks become clearer in the neural plate, we see the same nested pattern of expression described above for the 5-somite stage. If there is a delay from the onset of expression of *deltaA* and *deltaD* in a given cell to the onset of expression of *deltaB*, it is unlikely to be more than an hour or so.

deltaB expression persists longer in relation to the onset of cell differentiation than does *Delta1* in tetrapods, since it is coexpressed with *islet1* in developing primary neurons – something that is not seen in chick or *Xenopus* (Chitnis et al., 1995; Henrique et al., 1995 and unpublished observations). At later stages, however, a large proportion of *islet1*-expressing cells lack *deltaB* expression (data not shown), suggesting that *deltaB* does eventually switch off in these cells. As expected, expression of *deltaB* correlates with withdrawal from the cell cycle: in BrdU pulse-labelling experiments at the 5- to 6-somite stage (data not shown), 95% of the *deltaB*-expressing cells were unlabelled with BrdU, indicating that they had already finished their final S-phase (although not necessarily their final mitosis). Occasional *deltaB*-expressing cells (5%; 45/827 cells counted, 6 embryos) were, nevertheless, labelled with BrdU; similarly, a small proportion of *islet1*-expressing cells were labelled. Mitoses that are not completed until after neuronal commitment may explain why, although most primary neurons occur as isolated individuals, some are found in small clusters of two or three contiguous cells (see Fig. 5).

deltaA, *deltaB* and *deltaD* act together to single out the cells that become primary neurons

deltaA, *deltaB* and *deltaD* all show the characteristic signature of genes whose products deliver lateral inhibition: they are all normally expressed most strongly in the nascent neurons, yet the effect of artificially forcing expression at a high level in all the cells of a region of the neuroepithelium is to inhibit neurogenesis (Dornseifer et al., 1997; Appel and Eisen, 1998; and present data). Conversely, when the Delta-Notch pathway is blocked by an injection of *X-Delta1^{dn}* RNA, neurons are produced in excessive numbers and no longer as isolated individuals. The Mauthner cell provides a striking example: normally a single one is generated on each side of the brain, but when the Delta-Notch signalling pathway is blocked, several are produced in a cluster and, when the pathway is overactivated, none are produced (Fig. 7). The overproduction of Mauthner cells and of motor and sensory neurons seen after *X-Delta1^{dn}* RNA injection resembles that seen (in a more extreme form) in the *mindbomb* (*white tail*) neurogenic mutant (Jiang et al., 1996; Schier et al., 1996), reinforcing the suggestion that the *mindbomb* gene codes for a component of the Delta-Notch signalling pathway.

Since the product of each of the fish delta genes *deltaA*, *deltaB* and *deltaD* has an effect similar to that of X-Delta1 or NotchΔE, and since the Notch-binding extracellular domain is highly conserved in all of them, it seems very likely that each of them is capable of activating Notch. *deltaA*, *deltaB* and *deltaD* not only have products with similar activities; they are also themselves all regulated similarly in their expression by the level of activation of the Delta-Notch signalling pathway – activation of the pathway drives their expression down, and inactivation of the pathway allows their expression to rise. We infer that in zebrafish embryos the product of each of these three *delta* genes can act on neighbouring cells to repress its own expression and the expression of the other two also. This finding implies the existence of a positive feedback loop that will tend to amplify differences between adjacent cells, as explained in the Introduction; it implies also that the levels of expression of the three *delta* genes should all be raised or lowered together, as observed.

The regulation of *delta* expression in one cell by Delta activity in its neighbour creates a competitive relationship between the adjacent cells. Provided the regulatory effect is steep enough, so that a change in the Delta activity in one cell causes an even larger change of Delta activity in its neighbour, the feedback loop in the control of *delta* expression is sufficient to render unstable a uniform state where all cells express the genes and all inhibit one another, and to drive the system towards a state where some cells express the *delta* genes strongly and deliver inhibition, while their neighbours receive inhibition and express the genes only weakly (Collier et al., 1996). In this way, the demonstrated properties of *deltaA* and *deltaD*, with their widespread early expression, are sufficient to explain how, through competition among the cells of a neurogenic patch, isolated cells become singled out to develop as primary neurons, even if the cells in the patch are initially all equivalent.

deltaB may have a special role

There is, however, a proviso: we have not proved that the feedback effects for *deltaA* and *deltaD* are steep enough by themselves to destabilise the uniform state of mutual inhibition. *deltaB* may have a special role in this respect. Its expression – strong in the future neurons, undetectable in their neighbours – suggests that it is much more steeply sensitive to the level of Notch activation than is the expression of *deltaA*

or *deltaD*, and that it may thereby take the state of uniform mutual inhibition over the brink of instability and drive the emergence of isolated cells as winners of a competition based on lateral inhibition.

A Delta protein may exert effects not only by activating Notch, but also by interfering directly with the actions of other Delta proteins by binding to them (Fehon et al., 1990). Indeed, we have suggested elsewhere (Henrique et al., 1997a) that Delta1^{dn} may act in this way to make cells deaf to lateral inhibition: Delta1^{dn} may bind to and sequester endogenous Delta proteins on neighbouring cells, so that they are no longer available to activate Notch. It is possible that DeltaB in nascent neurons has a similar effect: by binding to the Delta proteins on neighbouring cells, it may help to protect the nascent neurons from lateral inhibition. Experiments to explore this possibility are in progress.

Other factors may bias the outcome of a Delta-Notch mediated competition

Although our findings imply that the selection of individual cells as primary neurons depends on a Delta-mediated competition, it remains possible that the competition is biased from the outset. Some cells may have an advantage over their neighours by virtue of higher starting levels of expression of proneural genes such as *achaete/scute* or *atonal* homologues (Cubas et al., 1991; Goriely et al., 1991), or larger endowments of proteins such as Numb (Zhong et al., 1996). In the early chick neural plate, for example, the initial level of expression of the *achaete/scute* homologue *Cash4* varies markedly from cell to cell in an apparently random fashion (Henrique et al., 1997b). Where two adjacent cells by chance both start with a similar strong advantage, both may progress to become committed to neuronal differentiation before competition mediated by lateral inhibition has time to be fought through to a conclusion that favours one cell over the other. A mechanism of this sort could account for the occasional groups of two or more contiguous primary neurons that are seen in the zebrafish (if these are not all the result of cell division, as discussed earlier, or of cell migration bringing cells into contact after they have become committed). Indeed, one can envisage a continuum of possibilities, from the extreme where the primary neurons are determined entirely by prior factors, with lateral inhibition acting subsequently and serving only to prevent undetermined neighbours of these cells from also becoming primary neurons, to the opposite extreme where all cells are equivalent at the outset and lateral inhibition with feedback is the all-important mechanism in making them different.

Differences between vertebrates and invertebrates in the control of neurogenesis may be less than they seem

We began this paper by pointing out a clash between the proposal, based on work in *Drosophila* and *C. elegans*, that cells within proneural clusters are singled out to become neurons by a competitive mechanism based on Delta-Notch signalling, and the observation in chick and *Xenopus* that *Delta1* is expressed only in the prospective neurons. It may be that these cells in chick and *Xenopus* are indeed specified by some prior mechanism, independent of *Delta* genes, as just discussed, and that Delta-Notch signalling serves only to prevent the neighbours of these predetermined cells from developing as neurons at the same time. Flies, tetrapods and fish may all control neurogenesis differently, and there may be important differences between primary and secondary neurogenesis. But the present observations on the zebrafish suggest at least two other possibilities. First, tetrapods may have other homologues of *Delta* or *Serrate*, yet to be characterised, that are expressed like the zebrafish *deltaA* or *deltaD* in all the cells of a neurogenic patch and mediate competitive lateral inhibition, with *Delta1* behaving like zebrafish *deltaB* to signal the outcome of the competition. Second, it is conceivable that in situ hybridisation may have given an incomplete picture of the expression of *Delta1* in tetrapods: we have argued that, in the fish, the low-level expression of *deltaA* and/or *deltaD* in cells other than the prospective primary neurons is functionally important, and there is no guarantee that in situ hybridisation is sensitive enough in all cases to detect levels of *delta* expression that are sufficient to activate Notch. The *Drosophila* paradigm, in which neurogenesis begins with a proneural cluster all of whose members express *Delta* and *Notch* and thereby compete for a neural fate, may therefore apply also, with minor variations, not just to the zebrafish but to vertebrates in general.

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