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Regulation of neuronal specification in the zebrafish spinal cord by Delta function

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SUMMARY

The vertebrate spinal cord consists of a large number of different cell types in close proximity to one another. The identities of these cells appear to be specified largely by information acquired from their local environments. We report here that local cell-cell interactions, mediated by zebrafish homologues of the *Drosophila melanogaster* neurogenic gene, *Delta*, regulate specification of diverse neuronal types in the ventral spinal cord. We describe identification of a novel zebrafish *Delta* gene expressed specifically in the nervous system and show, by expressing a dominant negative form of Delta protein in embryos, that Delta proteins mediate lateral inhibition in the zebrafish spinal cord. Furthermore, we find that Delta function is important for specification of a variety of spinal cord neurons, suggesting that lateral inhibition serves to diversify neuronal fate during development of the vertebrate spinal cord.

Key words: Lateral inhibition, Neurogenesis, Neurogenic genes, Pattern formation, Motoneurons

INTRODUCTION

During vertebrate neurogenesis, the neuroepithelium is patterned with the anterior region specified as brain and the posterior region as spinal cord. Subsequently, many distinct types of neurons differentiate at particular times and places within the developing central nervous system (CNS). The problem we address in this paper is how cells within the spinal cord are specified for different neuronal fates.

Specification, the process by which cellular identity is established during development (Kimmel et al., 1991), of ventral spinal cord cells results from inductive signaling. Axial mesoderm, which underlies the developing spinal cord and differentiates as notochord, induces neural tissue to form floorplate, a specialized cell type in the ventral midline (Placzek et al., 1990; Yamada et al., 1991). Both notochord and floorplate can induce formation of motoneurons (Yamada et al., 1991, 1993) and their absence, in mice and zebrafish, correlates with absence of motoneurons (Bovolenta and Dodd, 1991; Beattie et al., 1997). The signal for both the notochord and floorplate responsible for patterning ventral spinal cord appears to be Sonic Hedgehog (Shh), a secreted glycoprotein, which is initially expressed in axial mesoderm and later in floorplate and is necessary and sufficient to induce floorplate and motoneuron development (reviewed by Tanabe and Jessell, 1996).

Floorplate and motoneurons are just two of the diverse cell types in the ventral spinal cord and the question remains as to how this diversification is achieved. Shh might be a morphogen, since relatively high concentrations induce both floorplate and motoneurons, while lower concentrations induce only motoneurons (Roelink et al., 1995) and still lower concentrations induce interneurons dorsal to motoneurons (Ericson et al., 1997). Blockage of Shh signaling during a critical late phase of Shh requirement causes generation of interneurons rather than motoneurons, suggesting that different neuronal types have different requirements for Shh signaling (Ericson et al., 1996). Thus, graded distribution of Shh within ventral spinal cord may instruct a variety of different fates. Sequential signaling may contribute to spinal cord patterning as motoneurons are required for differentiation of specific interneurons (Pfaff et al., 1996).

We are investigating whether interactions among prospective neurons in ventral spinal cord are important for their specification by testing neuronal specification in zebrafish embryos, in which distinct neurons develop at characteristic positions and times (Kimmel and Westerfield, 1990). For example, motoneurons can be categorized as primary and secondary (Myers, 1985). Primary motoneurons are born before secondary motoneurons, are larger and fewer in number, and are spared in *ned-1* mutant embryos in which secondary motoneurons degenerate (Grunwald et al., 1988). Individual primary motoneurons can be identified by differences in cell body position, axon projection and gene expression (Eisen et al., 1986; Inoue et al., 1994; Appel et al., 1995; Tokumoto et al., 1995). Additionally, various types of

individually identifiable interneurons develop in the ventral spinal cord close to motoneurons (Bernhardt et al., 1990, 1992).

In *Drosophila melanogaster*, cellular interactions, mediated by proteins encoded by the so-called neurogenic genes *Notch* and *Delta*, operate to specify cells for different fates. For example, *Notch* and *Delta* are required to specify appropriate numbers of neuroblasts and epidermoblasts from ventral neuroectoderm (reviewed by Campos-Ortega, 1995). In the absence of *Notch* or *Delta* function, too many neuroblasts develop at the expense of epidermoblasts. Delta and Notch appear to function as ligand and receptor, respectively (Muskavitch, 1994). In a process called lateral inhibition (Simpson, 1990) or lateral specification (Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995; but see Muskavitch, 1994), high levels of Delta expression in specified neuroblasts are thought to activate Notch in neighboring cells, causing them to develop as epidermoblasts rather than neuroblasts (Campos-Ortega, 1995).

Notch and *Delta* homologues have been identified in *Caenorhabditis elegans* and a variety of vertebrate species (Artavanis-Tsakonas et al., 1995). Notch proteins that are constitutively active in the absence of ligand suppress neurogenic and myogenic developmental programs in cell culture and vertebrate embryos (Coffman et al., 1993; Kopan et al., 1994; Nye et al., 1994; Lardelli et al., 1996) as does overexpression of a full-length Delta protein (Chitnis et al., 1995). Conversely, expression of a dominant negative (dn) form of Delta in *Xenopus laevis* embryos causes too many cells to adopt neuronal fates (Chitnis et al., 1995). These observations are consistent with a role for vertebrate Delta and Notch proteins in mediating lateral inhibition, suggesting this is a conserved mechanism for specifying cells for different fates.

In *D. melanogaster*, neuroectodermal cells have either neural or epidermal fates. In contrast, all cells within the vertebrate neuroectoderm are destined for neural development, either as neurons or glia. Thus, lateral inhibition could regulate specification of neural cells as different types of neurons and glia. Although work in *X. laevis* showed that Delta-mediated lateral inhibition is important for controlling the number of neural plate cells that develop as neurons (Chitnis et al., 1995), the role of lateral inhibition in regulating neuronal identity in the developing spinal cord is unknown. We have extended previous work by testing how lateral inhibition contributes to specification of identified spinal cord neurons in zebrafish embryos. We report identification of a novel zebrafish *Delta* homologue, *deltaA*, expressed specifically in developing nervous system. *deltaA* is transiently expressed in cells specified for neuronal fates. Overexpression of full-length DeltaA suppresses neuronal specification and expression of a dn Delta causes too many cells to adopt neuronal fates. By examining the effect of dn Delta on neuronal identity, we find evidence that lateral inhibition is important for specification of different types of motoneurons and interneurons in the ventral spinal cord. Furthermore, our results suggest that in the ventral spinal cord, as in the *D. melanogaster* and vertebrate retinas (Cagan and Ready, 1989; Fortini et al., 1993; Austin et al., 1995; Dorsky et al., 1995, 1997), the time at which cells are competent to respond to inductive signals influences their specification for particular neuronal fates.

MATERIALS AND METHODS

Isolation of zebrafish *delta* genes

Approximately 1×10^6 plaques of a lamba ZAP-II cDNA library constructed from 15-19 h (hours at 28.5°C) zebrafish embryos were screened at low stringency using a *X. laevis X-Delta-1* cDNA (Chitnis et al., 1995; gift of C. Kintner) as probe to isolate zebrafish *delta* cDNAs. cDNA inserts were subcloned by helper phage cotransfection (Stratagene) and restriction enzyme mapping revealed two classes of cDNA, *deltaA*, described here, and *deltaD* (see Haddon et al., 1998). The longest representative of each class was sequenced, on both strands, with a Perkin-Elmer Applied Biosystems Automated DNA Sequencer Model 377 using FS dye terminator chemistry. Sequences were assembled and analyzed using the DNASTAR Lasergene package. The GenBank accession number for *deltaA* is AF030031.

Embryos

Embryos from the University of Oregon laboratory colony were raised at 28.5°C. Staging was according to Kimmel et al. (1995).

RNA in situ hybridization and immunohistochemistry

In situ RNA hybridization was conducted as described previously (Thisse et al., 1993) except that the probes were not hydrolyzed. For RNA-antibody double-labeling experiments, embryos were fixed in 4% paraformaldehyde and first processed for antibody labeling using α-Isl mAb 39.4D5 (Tsuchida et al., 1994), which was obtained from the Developmental Studies Hybridoma Bank, followed by the peroxidase anti-peroxidase antibody system (Sternberger Monoclonals, Inc.). A brown precipitate was formed by incubating embryos in 0.5 mg/ml diaminobenzidine (DAB) and 0.001% H_2O_2. Embryos were then refixed and processed for in situ RNA hybridization. Embryos were mounted in glycerol and photographed using a Zeiss Axioplan and Kodak T160 film. For additional antibody labeling experiments, the following primary antibodies were used: rabbit anti-Hu (gift of Linda Hansen and Jim Weston), zn5 (Trevarrow et al., 1990) and rabbit anti-GABA (Sigma). Fluorescent secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. Fluorescent images were collected using a Zeiss LSM 310 confocal microscope, assembled with VoxelView (Vital Images) and processed using Adobe Photoshop.

Expression constructs and RNA injections

A *Bam*HI-*Xho*I fragment of *deltaA* cDNA was subcloned into the CS2 vector (Turner and Weintraub, 1994; gift of D. Turner). This subclone eliminates the first methionine translation start codon of the long open reading frame and is predicted to encode an extracellular domain similar in size and sequence to other vertebrate Delta proteins (see Results and Haddon et al., 1998). CS2-*X-Delta-1*^{STU} (Chitnis et al., 1995) was a gift of C. Kintner and CS2-nucβ-gal was a gift of D. Turner. Plasmids were linearized with *Not*I and capped mRNA was synthesized using SP6 RNA polymerase and the mCAP kit (Stratagene). The integrity of in vitro synthesized mRNA was assessed using ethidium-agarose gel electorophoresis and the concentration determined by spectrophotometry.

deltaA and *X-Delta-1*^{STU} mRNA at 50-300 ng/μl and *lacZ* mRNA at 300 ng/μl were mixed with Phenol Red (Sigma), to allow visualization of injections, at a concentration of 0.01%. Injections of approx. 5 nl were made, using air pressure, into 1- to 8-cell-stage embryos. Injection was into the center of the yolk, from which cytoplasmic streaming distributes mRNA to all cells, or directly into a single cell. This restricts distribution of mRNA to descendants of the injected cell, representing a subset of all embryonic cells, as revealed by staining for β-galactosidase activity. Often, mRNA is restricted to the left or right side of the embryo. β-galactosidase activity was detected after fixing embryos in 4% paraformaldehyde for 1 hour at room temperature or overnight at 4°C by incubation in

4% 5-bromo-4-chloro-indoxyl-β-D-galactoside, 150 mM NaCl, 1mM $MgCl_2$, 1.5 mM $K_4[Fe_3(CN)_6]$, 1.5 mM $K_3[Fe_2(CN)_6]$, in 5 mM sodium phosphate buffer (pH 7.3) at 37°C. Alternatively, β-galactosidase was detected using an anti-β-galactosidase antibody (Promega).

RESULTS

deltaA is expressed during neuronal specification

Using a probe synthesized from *X. laevis X-Delta-1* (Chitnis et al., 1995) at low stringency, we cloned two zebrafish *delta* genes, *deltaA*, described here, and *deltaD*. The structures of *deltaA* and *deltaD* are presented more fully in an accompanying paper (Haddon et al., 1998) and *deltaD* has been recently described (Dornseifer et al., 1997). Briefly, both genes are predicted to encode proteins having large extracellular domains as well as transmembrane and intracellular domains. The putative extracellular domain of each protein is highly similar to those of Delta-like proteins identified in fly, nematode and other vertebrates as each has the DSL domain and EGF-like repeats characteristic of this group (Artavanis-Tsakonas et al., 1995; Henrique et al., 1995). If translation is assumed to initiate at the first methionine codon of the long open reading frame, then *deltaA* is predicted to encode a protein of 803 amino acids. Alignment with other Delta proteins reveals that DeltaA would have an additional 38 N-terminal amino acids (see Haddon et al., 1998). However, the nucleotide sequence at this putative start site, ACCAATG, does not match a consensus translation initiation sequence, CANCATG, assembled for vertebrates (Cavener, 1987). In contrast, the sequence at the second in-frame methionine codon, CATCATG, matches the consensus translation start sequence. Translation initiation at this site would eliminate the 38-amino-acid N-terminal extension, resulting in an extracellular domain equivalent in size to those of other Delta proteins. The intracellular domains are quite diverged. The DeltaA intracellular domain is 58 amino acids longer than that of DeltaD, which makes it similar in size to the *D. melanogaster* Delta intracellular domain. However, these sequences have little similarity to one another (see Haddon et al., 1998).

We examined the expression patterns of *deltaA* and *deltaD* by in situ RNA hybridization. *deltaA* expression is initiated in the neuroectoderm before that of *deltaD* (data not shown). We focus here on *deltaA* expression in the developing trunk neural plate and neural tube. In zebrafish, cells that give rise to primary neurons of the trunk begin to exit the mitotic cycle as gastrulation is completed (Kimmel and Westerfield, 1990). The first of these known to differentiate are primary sensory Rohon Beard neurons (RBs) (Lamborghini, 1980; Bernhardt et al., 1990) and primary motoneurons (Myers, 1985), which arise from lateral and medial regions of the neural plate, respectively. *deltaA* expression is initiated in the epiblast prior to completion of gastrulation (not shown). At the 2- to 3-somite stage (10.5 h) low levels of *deltaA* RNA are distributed throughout the trunk CNS, with cells expressing higher levels found in the medial and lateral regions of the neural plate (Fig. 1A). These regions correspond to the positions at which primary motoneurons and RBs originate. Cells expressing high levels of *deltaA* RNA do not form contiguous domains. Rather, single cells or small clusters of several cells showing high expression are interspersed with cells having lower expression. *deltaA* expression is specific to the developing nervous system (Fig. 1B,C), and continues to be expressed broadly in the CNS throughout neurogenesis (Figs 1C, 2E). The expression of *deltaA* falls within the spatial and temporal expression profile described for zebrafish *notch* genes (Bierkamp and Campos Ortega, 1993; Westin and Lardelli, 1997). The correlation of *deltaA* and *notch* expression is consistent with the possibility that in zebrafish, as in other animal embryos, Delta and Notch proteins interact to regulate specification of cell fate.

Double labeling shows that *deltaA* is expressed in cells specified for neuronal fates. Soon after gastrulation is completed, and 6-8 hours before axogenesis, primary motoneurons and RBs are marked by an antibody raised against rat Islet-1 protein (α-Isl) (Korzh et al., 1993). We refer to these cells, marked by gene expression prior to axogenesis, as presumptive primary motoneurons (pPMNs) and presumptive Rohon-Beard neurons (pRBs). We first detect α-Isl reactivity in pPMNs and pRBs at about the 2- to 3-somite stage. Fig. 2A shows a dorsal view of the neural keel, at the position of the third and fourth somites of a 4-somite stage (11.3 h) embryo probed for Isl protein and *deltaA* message. α-Isl-reactive pPMNs express high levels of *deltaA* RNA. In slightly older embryos, most pPMNs at the same position no longer express *deltaA* RNA (Fig. 2B) while more posterior, later born pPMNs do (data not shown). Similarly, at early stages pRBs express *deltaA* RNA (Fig. 2C) but later, at the same axial position, most do not (Fig. 2D). These observations suggest that *deltaA* is rapidly downregulated following neuronal specification. Cells that express only *deltaA* RNA are also evident in medial and lateral neural plate. This may result from *deltaA* expression preceding Isl expression and from *deltaA* expression in cells specified for other neuronal fates. At 24 h, α-Isl labels cells in ventrolateral spinal cord that are probably fated to become secondary motoneurons (Appel et al., 1995). Many, but not all, of these cells express *deltaA*, consistent with the proposed transient nature of *deltaA* expression (Fig. 2E). At 24 h, and throughout later embryogenesis, *deltaA* is broadly expressed in the spinal cord, suggesting that it is expressed by many types of cells (Fig. 2E; data not shown). We conclude that *deltaA* is expressed as neuronal specification occurs and subsequently downregulated in cells that have acquired specific neuronal fates.

Delta function regulates neuronal specification

Recent studies suggest that vertebrate *Delta* and *Notch* genes regulate neurogenesis (Coffman et al., 1993; Austin et al., 1995; Chitnis et al., 1995; Dorsky et al., 1995, 1997; Dornseifer et al., 1997). To initiate our investigation into the function of *delta* genes in neuronal specification, we tested the effects of full-length DeltaA protein and a truncated form of *X. laevis* Delta-1 protein (dnDelta), which acts in dominant negative fashion (Chitnis et al., 1995), on neurogenesis in zebrafish using a probe for *huC* RNA as a marker of cells undergoing neuronal differentiation (Kim et al., 1996). Fig. 3A shows the expression pattern of *huC* in a 6-somite-stage control embryo injected, at early cleavage stage, with *lacZ*

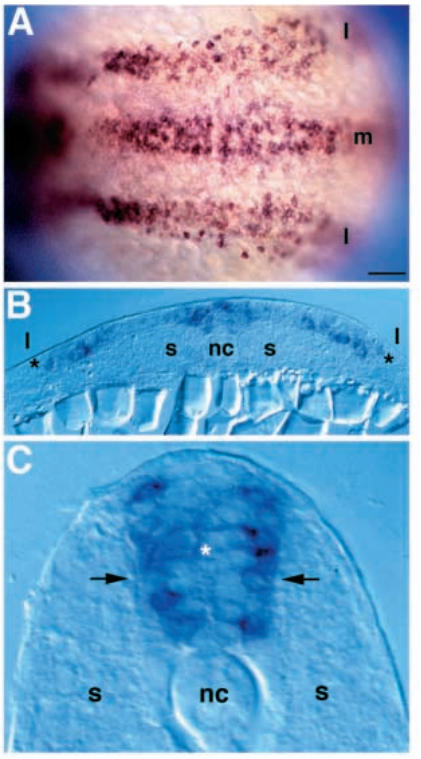

Fig. 1. *deltaA* expression in trunk neural plate and later neural tube. (A) Dorsal view focused on trunk of a 2- to 3-somite-stage embryo, anterior left, hybridized with digoxygenin-labeled antisense *deltaA* RNA. High levels of expression are revealed in medial (m) and lateral (l) regions of neural plate. (B) Transverse section through trunk of a 3-somite-stage embryo. Expression is specific to neuroepithelium (between asterisks). (C) Transverse section through trunk of a 20-somite-stage embryo. Cells expressing high levels of *deltaA* message are largely located near the neural tube basal surface (arrows) but are also in apically positioned cells (asterisk). nc, notochord; s, somitic mesoderm. Scale bar, 40 μm (A), 20 μm (B), 10 μm (C).

mRNA, which we use as a lineage tracer to reveal distribution of injected mRNAs. *huC* is expressed in discontinuous rows of cells in medial and lateral neural keel showing that some, but not all, cells in these regions are specified for neuronal development at this stage. Embryos coinjected with mRNAs encoding β-galactosidase and full-length DeltaA protein have fewer *huC*-expressing cells coincident with distribution of lineage tracer (Fig. 3B; Table 1), suggesting that high levels of DeltaA activity inhibit neurogenesis. At later stages of development, *deltaA*-injected embryos have fewer than normal secondary motoneurons, suggesting that *deltaA* overexpression generally inhibits neuronal specification (data not shown). In contrast, embryos injected with mRNA encoding dnDelta show an increased density of cells expressing *huC* spanning the normal spatial domains of *huC* expression (Fig. 3C; Table 1). To test if dnDelta interferes with DeltaA function, we injected both mRNAs together. In most embryos, the DeltaA inhibition of neurogenesis was suppressed, resulting in near normal or excess number of neurons (Table 1). Thus, dnDelta appears to disrupt DeltaA function. These data suggest that DeltaA, expressed in cells that have acquired neuronal fate, inhibits neuronal specification in neighboring cells. Disruption of Delta signaling activity, by dnDelta expression, results in failure of this lateral inhibition leading to specification of neuronal fate in excess cells. These observations are similar to those obtained by misexpression of full length and dominant negative X-Delta-1 proteins in *X. laevis* embryos (Chitnis et al., 1995).

Disruption of Delta-mediated lateral inhibition alters neuronal fates

Excess ventral spinal cord neurons have primary motoneuronal identity

To learn if Delta-mediated lateral inhibition regulates specification of neuronal identity in the spinal cord, we examined embryos expressing dnDelta with markers specific

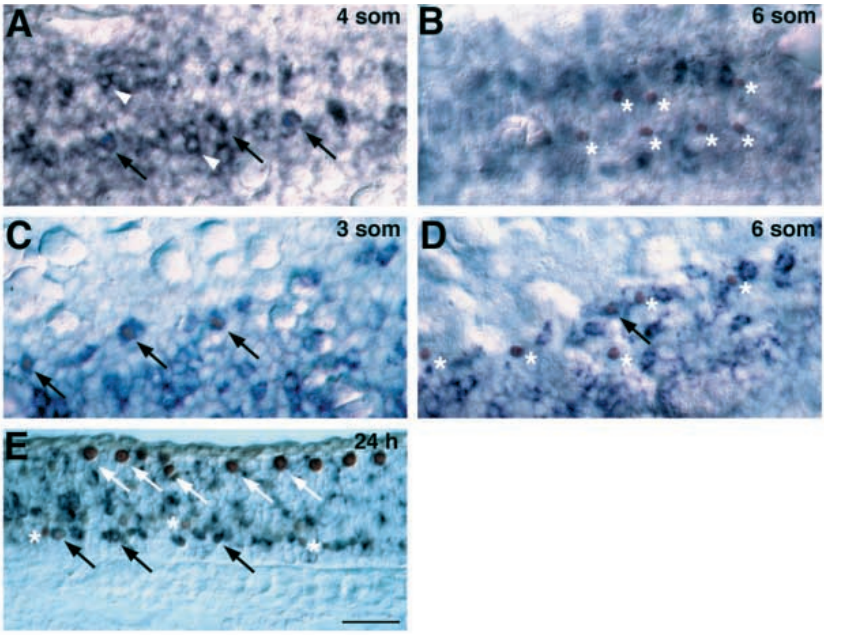

Fig. 2. *deltaA* is transiently expressed in cells specified for neuronal fate. Embryos labeled for nuclear Isl protein (brown), revealing pPMNs and pRBs, and *deltaA* RNA (blue). (A) Dorsal view of medial neural keel, at the position of the third and fourth somites, at the 4-somite stage (11.3 h). Doubly labeled cells (arrows) indicating pPMNs expressing *deltaA*. Many cells expressing *deltaA* at high level do not express Isl (white arrowheads). (B) Similar view to A, at the 6-somite stage. pPMNs (asterisks) do not express *deltaA* and lie ventral to most cells expressing *deltaA* at high level. (C) Dorsal view of lateral neural plate, at the position of the 2nd and 3rd somites, of a 3-somite-stage embryo. Doubly labeled pRBs are indicated by arrows. (D) Similar view to C of a 6-somite-stage embryo. Most pPMNS (asterisks) no longer express *deltaA* RNA. A single doubly labeled cell is indicated by the arrow. (E) Saggital section of 24 h spinal cord. Some presumptive secondary motoneurons express *deltaA* RNA (black arrows) while others do not (asterisks). RBs (white arrows) do not express *deltaA* at this stage. Scale bar, 25 μm.

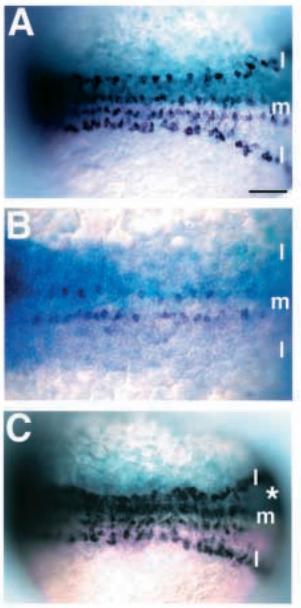

Fig. 3. Delta function regulates neurogenesis. Dorsal views (anterior left) of 6-somite-stage embryos injected with mRNA at early blastula stages and probed for *huC* expression. In each case, β-galactosidase activity, as lineage tracer, is revealed as light blue staining and *huC* expression as dark blue. (A) Embryo injected with *nlacZ* mRNA only. *huC* is expressed in discontinuous, bilateral rows of cells in medial (m) and lateral (l) regions of neural keel. (B) Embryo injected with full-length *deltaA* and *nlacZ* mRNAs. Lineage tracer is distributed thoughout the embryo, with the right side (upper portion) receiving more than the left (lower). *huC* expression is absent from both lateral domains and the number of *huC*-expressing cells in the medial rows is reduced, particularly on the right side. Lateral domains seem more sensitive than medial domains to increased *deltaA* expression (two independent experiments: 40% and 47% of embryos with decreased medial *huC* expression while greater than 90% of embryos show reduced lateral *huC* expression). (C) Embryo co-injected with *nlacZ* and *X-Delta-1*^{STU} mRNA. Lineage tracer shows that the right side (asterisk) received most of the injected mRNA. The density of *huC*-labeled neurons is increased in medial and lateral regions of neural keel receiving injected mRNA. Scale bar, 50 μm.

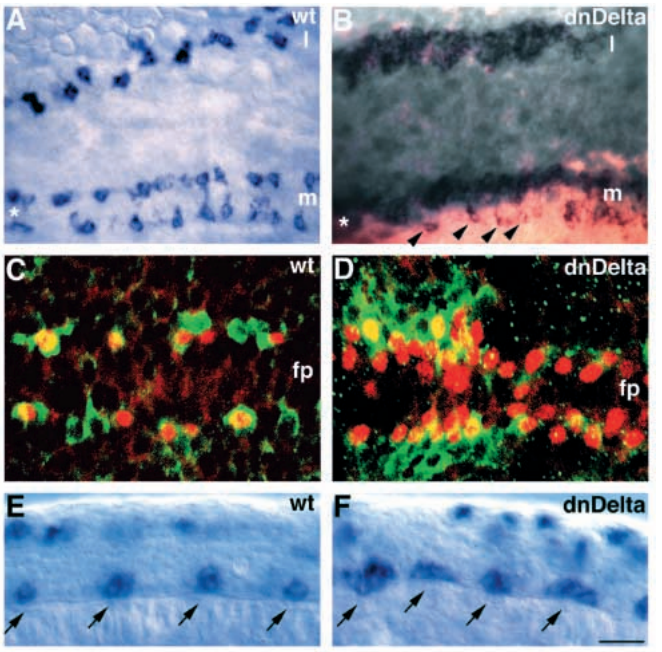

Fig. 4. Disruption of Delta function causes overproduction of primary motoneurons. (A) Dorsal view of neural plate of an uninjected 4-somite-stage embryo showing *islet1* expression by in situ RNA hybridization in medial (m) and lateral (l) regions. *islet1* expression marks pPMNs medially (two longitudinal rows bordering the floorplate, marked by an asterisk) and pRBs laterally. (B) Dorsal view of 4-somite-stage embryo injected with *X-Delta-1*^{STU} and *lacZ* mRNA and probed for *islet1* expression. Excess pPMNs and pRBs develop medially and laterally, respectively, on the side receiving most injected mRNA. Arrowheads indicate distribution of motoneurons, which is similar to that of uninjected embryos, on the side receiving little mRNA. Asterisk marks position of floorplate between motoneuron rows. (C) Confocal image, dorsal view, of uninjected 6-somite-stage embryo labeled with α-Isl (red) and α-Hu (green) antibodies. α-Isl reactivity precedes that of α-Hu (data not shown); thus, α-Isl^{+} motoneurons flanking the floorplate (fp) are sometimes unlabeled by α-Hu. Several non-motoneurons (α-Hu-positive, α-Isl-negative) are interspersed among motoneurons. (D) Confocal image, dorsal view, of 6-somite-stage embryo injected with *X-Delta-1*^{STU} mRNA and labeled with α-Isl (red) and α-Hu (green). Nearly every cell flanking the floorplate is a motoneuron. Some motoneurons also develop just dorsal to the floorplate. (E) Wild-type expression pattern of *islet2* RNA at 16-somite-stage, shown in lateral view. *islet2* is expressed in CaP and VaP motoneurons (arrows). Labeled cells in dorsal spinal cord are pRBs. (F) Similarly staged embryo injected with *X-Delta-1*^{STU} mRNA and probed for *islet2* expression. *islet2* is expressed in clusters of cells located at the normal positions of CaP and VaP motoneurons (arrows). Scale bar, 20 μm (A,B), 10 μm (C,D) and 15 μm (E,F).

for ventral neural tube fates. First, we found that neural-plate-stage embryos have an increased number of medially located cells expressing *islet1* RNA, a marker of motoneuronal fate (Inoue et al., 1994; Appel et al., 1995) (Fig. 4A,B). Next, we determined the extent to which ventral spinal cord cells are caused to adopt motoneuronal fates when Delta function is disrupted. Fig. 4C shows an uninjected 6-somite-stage embryo probed with α-Isl, to label presumptive motoneurons, and α-Hu, which labels all differentiating neurons (Marusich et al., 1994) to reveal the early pattern of neuronal specification. pPMNs form two single-cell-width columns flanking the floorplate. Within these columns, pPMNs are arranged in a regular, periodic fashion (Appel et al., 1995). α-Isl reactivity precedes that of α-Hu; thus, Isl-positive cells are occasionally unlabeled by α-Hu. Some cells are Hu-positive but Isl-negative; other cells express neither marker. This suggests that, during early stages of neurogenesis, the developing trunk ventral neural keel of zebrafish includes cells specified for primary motoneuronal fate (Isl^{+}Hu^{+}), cells specified as neurons but for non-motoneuronal fates (Isl^{-}Hu^{+}) and cells that are not yet specified as neurons (Isl^{-}Hu^{-}). In contrast, in embryos expressing dnDelta, all neuronal cells near the floorplate are Isl-positive and thus pPMNs (Fig. 4D). These cells are limited to rows one or two cells wide bordering the floorplate. α-Hu labeling shows that dnDelta also causes overproduction of neuronal cells more distant from floorplate; however, these cells are unlabeled by α-Isl and,

Table 1. Effect of injected synthetic mRNAs

mRNA injected	Total embryos injected	Reduced neurons (%)	Increased neurons (%)	No change (%)
deltaA	179	140 (78)	0	39 (22)
X-Delta-1^{STU}	139	0	116 (83)	23 (17)
deltaA/X-Delta-1^{STU}	104	4 (4)	75 (72)	25 (24)
nlacZ	43	9 (21)	0	34 (79)

Embryos were injected and processed as described in Materials and methods. Results expressed as number and, in parentheses, percentage of total of embryos showing changes in *huC* labeled primary neurons. For coinjection experiments, *deltaA* mRNA at a concentration that reduces the number of neurons (approximately 100 ng/μl) was mixed with *X-Delta-1^{STU}* mRNA at an equal or slight excess molar concentration.

thus, are not motoneurons. These observations indicate that Delta function is required to establish the appropriate number and distribution of primary motoneurons. Disruption of Delta function results in formation of supernumerary motoneurons, which appear to arise at the expense of other cell types that are normally interspersed among them.

To learn the identities of motoneurons generated when Delta function is perturbed, we examined *islet2* expression, which normally marks only one or two specific primary motoneurons per hemisegment, CaP and VaP (Appel et al., 1995) (Fig. 4E). In contrast, we find clusters of 3-7 *islet2*-positive cells in the ventral spinal cords of *X-Delta-1^{STU}*-injected embryos (Fig. 4F), suggesting that failure of lateral inhibition leads to overproduction of primary motoneurons. Furthermore, *islet2*-positive cells are limited to clusters midway between somite boundaries at the positions normally occupied by CaP and VaP motoneurons. This shows that, as revealed by *islet2* expression, supernumerary primary motoneurons acquire identities appropriate for their location, supporting our previous results showing that spinal cord gene expression and motoneuronal identity are positionally specified (Eisen, 1991; Appel et al., 1995). Additionally, this result implies that the positional information that specifies identity of individual primary motoneurons is intact in the absence of normal Delta function. A similar conclusion has been drawn for neuroblast specification in flies, in which it was shown that the identity of neuroblasts in the peripheral nervous system is independent of the neurogenic loci (Goriely et al., 1991).

Other types of ventral spinal cord neurons are reduced when Delta function is perturbed

We examined the effect of dnDelta expression on development of secondary motoneurons using the zn5 monoclonal antibody (Trevarrow et al., 1990), which recognizes the DMGRASP protein (Fashena, 1996). In control embryos, zn5 labels secondary motoneuron cell bodies and axons (Fashena, 1996) (Fig. 5A). dnDelta expression greatly reduces the number of secondary motoneurons in embryos showing widespread distribution of lineage tracer in ventral spinal cord (28 of 40 embryos). Fig. 5B shows an embryo in which lineage tracer was distributed to clusters of cells in the spinal cord. zn5 labeling is evident outside these clusters but is largely absent from cells expressing lineage tracer. Additionally, the ventral roots appear to be reduced in size, suggesting that fewer secondary motoneuron axons contributed to the nerve. Thus, embryos expressing dnDelta have too many primary motoneurons and too few secondary motoneurons. One possible explanation for this finding is that cells normally specified as secondary motoneurons develop as primary motoneurons in the absence of Delta function.

Two types of interneurons, KA and VeLD, positioned close to motoneurons (Fig. 6A), can be identified with antibody specific to the neurotransmitter GABA (Bernhardt et al., 1992). KA neurons lie adjacent to the floorplate, ventromedial to motoneurons. VeLD neurons are more dorsal, intermixed with motoneurons. In *X-Delta-1^{STU}*-injected embryos with lineage tracer distributed broadly throughout the ventral spinal cord, KA and VeLD interneurons are reduced in number or absent (7 of 16 embryos; Fig. 6B). Thus, disruption of Delta-mediated lateral inhibition results in overproduction of primary motoneurons with corresponding decreases in secondary motoneurons, KA interneurons and VeLD interneurons. These observations indicate that Delta function is required for specification of a variety of neuronal fates in zebrafish ventral spinal cord.

DISCUSSION

To learn how different kinds of neurons are specified in the ventral spinal cord, we asked two questions. First, do zebrafish neurons influence the fates of neighboring cells through Delta/Notch-mediated lateral inhibition? Second, what is the consequence for ventral spinal cord cell fates when lateral inhibition is disrupted?

Zebrafish *delta* genes encode lateral inhibitory signals

The lateral inhibition model stems from observations of bristle formation on the cuticle of *Rhodnius prolixus* made by Wigglesworth (1940), who concluded that bristles prevent formation of similar structures within a certain radius. When bristles were removed, they were regenerated by cells that would not normally give rise to bristles, suggesting that many cells have potential for bristle development but that, typically, only a subset of cells express this potential. Interactions which specify equivalent cells for different fates are now recognized as an important patterning mechanism (Greenwald and Rubin, 1992). In *D. melanogaster*, lateral inhibition operates to specify cells for neuroblast or epidermoblast fates in the embryonic CNS and the adult PNS; in the absence of lateral inhibition, mediated by neurogenic genes, too many neuroblasts develop at the expense of

epidermoblasts (reviewed by Simpson, 1990; Campos-Ortega, 1995). A simple prediction of the lateral inhibition model is that the neurogenic gene *Delta*, which functions cell-nonautonomously, is expressed at higher levels in neural cells than in neighboring non-neural cells. Indeed, genetic mosaic analysis indicated that cells tend to be specified for neural fate when they have a greater dosage of *Delta* relative to adjacent cells (Heitzler and Simpson, 1991). However, Delta protein is expressed uniformly throughout cell populations undergoing fate specification and is not evident in delaminated neuroblasts (Kooh et al., 1993). This was taken as evidence to support a model of mutual inhibition (Goriely et al., 1991) in which every cell of an equivalence group is prevented from adopting a neural fate and only those cells that escape the inhibitory field undergo neural development (Muskavitch, 1994).

Zebrafish *deltaA* is expressed broadly throughout the neural plate and, later, spinal cord. However, this expression is nonuniform as some cells express *deltaA* at higher levels than others. Double labeling shows that early induced cells specified for primary motoneuron and RB fates express high levels of *deltaA*, and that *deltaA* is later expressed by cells specified for secondary motoneuron fate as well as by other types of cells. *deltaA* expression appears to be quickly downregulated in these cells soon after their birth. Thus, in many ways, expression of *deltaA* is consistent with predictions of the lateral inhibition model. In actuality, the patterns of deltaA expression and neuronal differentiation do not conform precisely to the model. In many instances, adjacent cells express high levels of *deltaA*. Likewise, adjacent cells may develop as primary motoneurons or RBs (see Figs 1A, 3A, 4C). Perhaps, as a result of cell movements during neurogenesis, cells that are specified for neuronal fates are brought into contact with one another. Additionally, in zebrafish, some cells may be specified for neuronal fates before completion of mitosis (B. Appel and K. Stoesser, unpublished results; Haddon et al., 1998). Thus, small clusters of adjacent neurons might arise, circumventing lateral inhibition of similar fate (see Haddon et al., 1998).

To date, four zebrafish *Notch* homologues have been described (Bierkamp and Campos-Ortega, 1993; Westin and Lardelli, 1997). Although we have not directly compared them, the expression patterns of zebrafish *notch* genes and *deltaA* appear to coincide within the developing nervous system. Thus, zebrafish *notch* and *delta* genes are expressed at the appropriate times and places to mediate lateral inhibition in the CNS.

If DeltaA acts as an inhibitory signal, increasing its level of expression should prevent neuronal specification. When we inject mRNA encoding full-length DeltaA protein into embryos, we find that the number of cells expressing a pan-neuronal marker is greatly reduced. This result is identical to that obtained when X-Delta-1 is overexpressed in frog embryos (Chitnis et al., 1995). Indeed, X-Delta-1 overexpression in zebrafish embryos also inhibits neuronal specification (B. Appel, unpublished observations) as does overexpression of zebrafish DeltaD (Dornseifer et al., 1997; B. Appel, unpublished observations). At least three zebrafish *delta* genes are expressed in the developing nervous system (this work; Dornseifer et al., 1997; Haddon et al., 1998). Thus, lateral inhibition may be mediated by multiple Delta family members. Comparison of *delta* gene expression patterns and identification of mutations that affect individual *delta* genes will reveal if these genes function within different subpopulations of cells in the CNS and whether they have similar or overlapping functions in the same cells.

Recently, two zebrafish mutations have been described, *whitetail* (Jiang et al., 1996) and *mind bomb* (Schier et al., 1996), which result in overproduction of early specified neurons in zebrafish. These phenotypes are similar to some of those we describe here arising from expression of dnDelta. Thus, the *whitetail* and *mind bomb* mutations, which are allelic, may identify a component of the Delta/Notch signaling pathway. It will be interesting to further compare the mutant phenotype with the dnDelta phenotype as well as to learn the identity of the affected gene.

Lateral inhibition may be required for specification of multiple ventral spinal cord neuronal fates

Neuronal diversity is often attained through a stereotypical sequence of cell differentiation. For example, in each ommatidium of the *D. melanogaster* retina, specific cells develop in a precise order. Inductive signaling is important for specification of these cell types and early differentiating cells appear to recruit later-differentiating cells to the ommatidium (Tomlinson and Ready, 1987). Inactivation of Notch function, by means of a temperature-sensitive *Notch* mutation, at progressively later times during retinal development affects the fates of progressively later-developing cells (Cagan and Ready, 1989), suggesting that Notch function is required as each fate is specified. Expression of a ligand-independent, activated Notch causes retinal cells to adopt inappropriate fates, perhaps as a consequence of delaying their differentiation (Fortini et al., 1993). Thus, Notch activity may regulate the ability of cells to respond to inductive signaling.

Likewise, in the vertebrate retina, different cell types develop in a particular sequence. Lineage analysis of retinal cells suggests that progenitors are multipotent and that environmental signals contribute to specification (reviewed by Cepko et al., 1996). Reduction of Notch activity increases the number of cells that adopt the earliest specified fate, that of retinal ganglion cells, while overexpression of Delta or of activated Notch reduces ganglion cell number (Austin et al., 1995). Consistent with the lateral inhibition model, when cells overexpressing X-Delta-1 have normal neighbors, they take earlier fates, while dominant negative X-Delta-1 causes nearly all expressing cells also to take the earliest specified fates of ganglion cells and cone photoreceptors (Dorsky et al., 1997). Together, these observations suggest that disruption of Delta/Notch signaling allows excess cells to be specified for early fates and that Delta and Notch functions normally regulate the number of retinal cells specified for any particular fate.

In *X. laevis* embryos, expression of activated Notch results in neural hypertrophy (Coffman et al., 1993). Notch activation appears to delay cell differentiation and an increased number of progenitor cells commit to neural development, leading to the proposal that Notch signaling regulates the number of cells that can respond to any given inductive signal.

In the zebrafish spinal cord, regulation of primary and secondary motoneuron specification fits well with this model. Primary and secondary motoneurons are born beginning about 9-10 h and 14-15 h, respectively (Myers et al., 1986), at least one cell cycle apart (Kimmel et al., 1994). Zebrafish *shh*

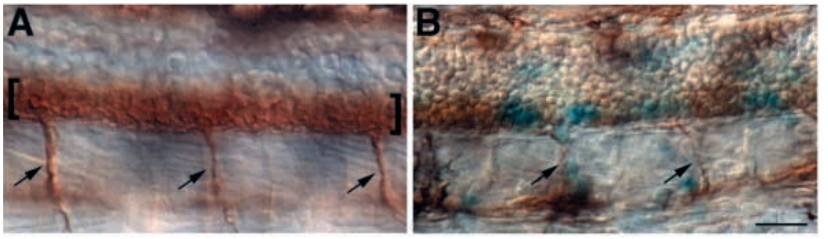

Fig. 5. Disruption of lateral inhibition reduces the number of secondary motoneurons. (A) Lateral view of a 36 h control embryo labeled with zn5, which identifies secondary motoneurons (brackets) and ventral nerves (arrows) exiting the spinal cord. (B) Similar view of a 36 h embryo injected with *X-Delta-1*^{STU} and *nlacZ* mRNAs. Lineage tracer (blue staining) is expressed in small clusters of spinal cord cells. Outside the clusters, zn5 labeling looks essentially normal. Cells within lineage-marked clusters are largely zn5-negative, showing that secondary motoneurons have not differentiated. Ventral nerves (arrows) appear reduced in size, suggesting the presence of fewer motor axons than in normal embryos. Scale bar, 20 μM.

expression is initiated in presumptive dorsal mesoderm at about 7 h (Krauss et al., 1993). In *cyclops; floating head* double mutant embryos, which lack differentiated notochord and floorplate, *shh* expression is initiated normally but not maintained after gastrulation and primary, but no secondary motoneurons develop (Beattie et al., 1997). Thus, early *shh* signaling may induce development of primary, but not secondary, motoneurons. In chick, motoneuronal induction requires exposure to Shh for 1-2 cell cycles (Ericson et al., 1996). The zebrafish cell cycle length during early neurogenesis is about 4 hours (Kimmel et al., 1994). Thus, secondary motoneurons are born 1-2 cell cycles after initiation of *shh* expression. Together, these observations suggest that secondary motoneurons are equivalent to chick motoneurons in their requirement for Shh signaling, but that primary motoneurons require only brief exposure. We have shown here that, in the absence of lateral inhibition, too many primary motoneurons develop concomitant with the loss of secondary motoneurons. Thus, we propose that Delta-Notch signaling specifies primary and secondary motoneuronal fates by regulating how neural precursor cells respond to Shh signaling. In this model, some cells of the neural plate respond to Shh by immediately developing as primary motoneurons. DeltaA is expressed at high levels in these cells and inhibits neighboring cells from responding to Shh in the same way. Later, as DeltaA is downregulated in primary motoneurons, neighboring cells are released from lateral inhibition and respond to Shh by adopting secondary motoneuronal fate. Alternatively, secondary motoneurons may be specified by a late-arising signal that acts with, or subsequent to, Shh. In either case, by controlling the number of cells that respond to inductive signals, Delta-mediated lateral inhibition facilitates specification of distinct types of motoneurons in the zebrafish spinal cord.

We do not know how specification of KA and VeLD interneurons fits into these models, as we know less about when and where they are born with respect to motoneurons. These neurons are anti-GABA reactive beginning at about 18 h (Bernhardt et al., 1992; B. Appel, unpublished observations). Thus, they may be born at the same time as primary motoneurons or the earliest secondary motoneurons. Perhaps Shh is able to specify a variety of fates by virtue of a cell's position within a Shh gradient as well by length of exposure to Shh (Ericson et al., 1996). Additionally, early differentiating neurons may express signals that instruct the fates of later-differentiating ones, a possibility demonstrated by the requirement of motoneurons for development of a specific interneuron in mice (Pfaff et al., 1996). Either way, Delta/Notch signaling, by mediating lateral inhibition, controls the number of cells competent to respond to any particular instructive signal, thus diversifying neuronal fates in the vertebrate ventral spinal cord.

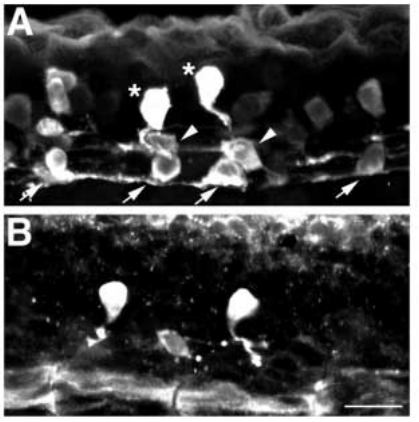

Fig. 6. Ventral interneurons are reduced in the absence of lateral inhibition. (A) Lateral view of 24 h spinal cord showing distribution of GABA-positive neurons. KA interneurons (arrows) differentiate in ventral spinal cord, VeLD interneurons (arrowheads) are slightly more dorsal and CoSA interneurons (asterisks) are dorsal to VeLDs and just ventral to RBs. (B) Lateral view of a 24 h embryo injected with *X-Delta-1*^{STU} and *nlacZ* mRNAs and labeled with anti-GABA. A single VeLD and two CoSA interneurons have differentiated. Scale bar, 10 μm.

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REFERENCES

Appel, B., Korzh, V., Glasgow, E., Thor, S., Edlund, T., Dawid, I. B. and Eisen, J. S. (1995). Motoneuron fate specification revealed by patterned LIM homeobox gene expression in zebrafish. *Development* **121,** 4117-4125.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268,** 225-232.

Austin, C. P., Feldman, D. E., Ida, J. A., Jr. and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* **121,** 3637-3650.

Beattie, C. E., Hatta, K., Halpern, M. E., Liu, H., Eisen, J. S. and Kimmel, C. B. (1997). Temporal separation in the specification of primary and secondary motoneurons in zebrafish. *Dev. Biol.* **187,** 171-182.

Bernhardt, R. R., Chitnis, A. B., Lindamer, L. and Kuwada, J. Y. (1990). Identification of spinal neurons in the embryonic and larval zebrafish. *J. Comp. Neurol.* **302,** 603-616.

Bernhardt, R. R., Patel, C. K., Wilson, S. W. and Kuwada, J. Y. (1992). Axonal trajectories and distribution of GABAergic spinal neurons in wildtype and mutant zebrafish lacking floor plate cells. *J. Comp. Neurol.* **326,** 263-272.

Bierkamp, C. and Campos-Ortega, J. A. (1993). A zebrafish homologue of the *Drosophila* neurogenic gene *Notch* and its pattern of transcription during early embryogenesis. *Mech. Dev.* **43,** 87-100.

Bovolenta, P. and Dodd, J. (1991). Perturbation of neuronal differentiation and axon guidance in the spinal cord of mouse embryos lacking a floor plate: analysis of Danforth's short-tail mutation. *Development* **113,** 625-639.

Cagan, R. L. and Ready, D. F. (1989). *Notch* is required for successive cell decisions in the developing *Drosophila* retina. *Genes Dev.* **3,** 1099-1112.

Campos-Ortega, J. A. (1995). Genetic mechanisms of early neurogenesis in *Drosophila melanogaster*. *Mol. Neurobiol.* **10,** 75-89.

Cavener, D. R. (1987). Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. *Nucleic Acids Res.* **15,** 1353-1361.

Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M and Ezzeddine, D. (1996). Cell fate determination in the vertebrate retina. *Proc. Nat. Acad. Sci. USA* **93,** 589-595.

Chitnis, A., Henrique, D., Lewis, J., Ish Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature* **375,** 761-766.

Coffman, C. R., Skoglund, P., Harris, W. A. and Kintner, C. R. (1993). Expression of an extracellular deletion of Xotch diverts cell fate in *Xenopus* embryos. *Cell* **73,** 659-671.

Dornseifer, P., Takke, C. and Campos-Ortega, J. A. (1997). Overexpression of a zebrafish homologue of the *Drosophila* neurogenic gene *Delta* perturbs differentiation of primary neurons and somite development. *Mech. Dev.* **63,** 159-172.

Dorsky, R. I., Chang, W. S., Rapaport, D. H. and Harris, W. A. (1997). Regulation of neuronal diversity in the *Xenopus* retina by Delta signalling. *Nature* **385,** 67-70.

Dorsky, R. I., Rapaport, D. H. and Harris, W. A. (1995). Xotch inhibits cell differentiation in the *Xenopus* retina. *Neuron* **14,** 487-496.

Eisen, J. S., Myers, P. Z. and Westerfield, M. (1986). Pathway selection by growth cones of identified motoneurones in live zebrafish embryos. *Nature* **320,** 269-271.

Eisen, J. S. (1991). Determination of primary motoneuron identity in developing zebrafish embryos. *Science* **252,** 569-572.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87,** 661-673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90,** 169-180.

Fashena, D. S. (1996). A neuron's reach can exceed its GRASP: expression of a cell adhesion molecule in the zebrafish embryo. PhD dissertation. University of Oregon.

Fortini, M. E., Rebay, I., Caron, L. A. and Artavanis Tsakonas, S. (1993). An activated Notch receptor blocks cell-fate commitment in the developing *Drosophila* eye. *Nature* **365,** 555-557.

Goriely, A., Dumont, N., Dambly Chaudiere, C. and Ghysen, A. (1991). The determination of sense organs in *Drosophila*: effect of the neurogenic mutations in the embryo. *Development* **113,** 1395-1404.

Greenwald, I. and Rubin, G. M. (1992). Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. *Cell* **68,** 271-281.

Grunwald, D. J., Kimmel, C. B., Westerfield, M., Walker, C. and Streisinger, G. (1988). A neural degeneration mutation that spares primary neurons in the zebrafish. *Dev. Biol.* **126,** 115-128.

Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D. and Lewis, J. (1998). Multiple *delta* genes and lateral inhibition in zebrafish primary neurogenesis. *Development* **125**, 359-370.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* **64,** 1083-1092.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish Horowicz, D. (1995). Expression of a *Delta* homologue in prospective neurons in the chick. *Nature* **375,** 787-790.

Inoue, A., Takahashi, M., Hatta, K., Hotta, Y. and Okamoto, H. (1994). Developmental regulation of *islet-1* mRNA expression during neuronal differentiation in embryonic zebrafish. *Dev. Dyn.* **199,** 1-11.

Jiang, Y. J., Brand, M., Heisenberg, C.-P., Beuchle, D., Furutani-Seiki, M., Kelsh, R. N., Warga, R. M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Mullins, M. C., Odenthal, J., van Eeden, F. J. M. and Nüsslein-Volhard, C. (1996). Mutations affecting neurogenesis and brain morphology in the zebrafish, *Danio rerio*. *Development* **123,** 205-216.

Kim, C. H., Ueshima, E., Muraoka, O., Tanaka, H., Yeo, S. Y., Huh, T. L. and Miki, N. (1996). Zebrafish *elav/HuC* homologue as a very early neuronal marker. *Neurosci. Lett.* **216,** 109-112.

Kimmel, C. B. and Westerfield, M. (1990). Primary neurons of the zebrafish. In *Signals and Sense: Local and Global Order in Perceptual Maps* (ed. G. M. Edelman and W. M. Cowan), pp. 561-588. Wiley Interscience, New York.

Kimmel, C. B., Kane, D. A. and Ho, R. K. (1991). Lineage Specification During Early Embryonic Development of the Zebrafish. In *Cell-Cell Interactions in Early Development* (ed. J. Gerhart), pp. 203-225. Wiley-Liss, Inc. New York.

Kimmel, C. B., Warga, R. M. and Kane, D. A. (1994). Cell cycles and clonal strings during formation of the zebrafish central nervous system. *Development* **120,** 265-276.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of Embryonic Development of the Zebrafish. *Dev. Dyn.* **203,** 253-310.

Kooh, P. J., Fehon, R. G. and Muskavitch, M. A. (1993). Implications of dynamic patterns of *Delta* and *Notch* expression for cellular interactions during *Drosophila* development. *Development* **117,** 493-507.

Kopan, R., Nye, J. S. and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120,** 2385-2396.

Korzh, V., Edlund, T. and Thor, S. (1993). Zebrafish primary neurons initiate expression of the LIM homeodomain protein Isl-1 at the end of gastrulation. *Development* **118,** 417-425.

Krauss, S., Concordet, J. P. and Ingham, P. W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75,** 1431-1444.

Lamborghini, J. E. (1980). Rohon-beard cells and other large neurons in *Xenopus* embryos originate during gastrulation. *J. Comp. Neurol.* **189,** 323-333.

Lardelli, M., Williams, R., Mitsiadis, T. and Lendahl, U. (1996). Expression of the Notch3 intracellular domain in mouse central nervous system progenitor cells is lethal and leads to disturbed neural tube development. *Mech. Dev.* **59,** 177-190.

Marusich. M. F., Furneaux, H. M., Henion, P. D. and Weston, J. A. (1994). Hu neuronal proteins are expressed in proliferating neurogenic cells. *J. Neurobiol.* **25,** 143-155.

Muskavitch, M. A. (1994). Delta-Notch signaling and *Drosophila* cell fate choice. *Dev. Biol.* **166,** 415-430.

Myers, P. Z. (1985). Spinal motoneurons of the larval zebrafish. *J. Comp. Neurol.* **236,** 555-561.

Myers, P. Z., Eisen, J. S. and Westerfield, M. (1986). Development and axonal outgrowth of identified motoneurons in the zebrafish. *J. Neurosci.* **6,** 2278-2289.

Nye, J. S., Kopan, R. and Axel, R. (1994). An activated Notch suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. *Development* **120,** 2421-2430.

Pfaff, S. L., Mendelsohn, M., Stewart, C. L., Edlund, T. and Jessell, T. M. (1996). Requirement for LIM homeobox gene *Isl1* in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. *Cell* **84,** 309-320.

Placzek, M., Tessier Lavigne, M., Yamada, T., Jessell, T. and Dodd, J. (1990). Mesodermal control of neural cell identity: floor plate induction by the notochord. *Science* **250,** 985-988.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81,** 445-455.

Schier, A. F., Neuhauss, S. C. F., Harvey, M., Malicki, J., Solnica-Kretzel, L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z., Yang, H. and Driever, W. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123,** 165-178.

Simpson, P. (1990). Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of *Drosophila*. *Development* **109,** 509-519.

Tanabe, Y. and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274,** 1115-1123.

Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H. (1993). Structure of the zebrafish *snail1* gene and its expression in wild-type, *spadetail* and *no tail* mutant embryos. *Development* **119,** 1203-1215.

Tokumoto, M., Gong, Z., Tsubokawa, T., Hew, C. L., Uyemura, K., Hotta, Y. and Okamoto, H. (1995). Molecular heterogeneity among primary motoneurons and within myotomes revealed by the differential mRNA expression of novel *islet-1* homologs in embryonic zebrafish. *Dev. Biol*. **171,** 578-589.

Tomlinson, A. and Ready, D. F. (1987). Neuronal differentiation in the *Drosophila* ommatidium. *Dev. Biol.* **120,** 366-376.

Trevarrow, B., Marks, D. L. and Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron* **4,** 669-679.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79,** 957-970.

Turner, D. L. and Weintraub, H. (1994). Expression of *achaete-scute homolog 3* in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8,** 1434-1447.

Westin, J. and Lardelli, M. (1997). Three novel *Notch* genes in zebrafish: implications for vertebrate *Notch* gene evolution and function. *Dev. Genes. Evol.* **207,** 51-63.

Wigglesworth, V. B. (1940). Local and general factors in the development of 'pattern' in *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol.* **17,** 180-200.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* **64,** 635-647.

Yamada, T., Pfaff, S. L., Edlund, T. and Jessell, T. M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. *Cell* **73,** 673-686.