SEARCHING FOR INFLUENCES ON NEURAL CREST DEVELOPMENT IN ZEBRAFISH

by

Tara Joy Massad

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Dr. Judith S. Eisen

An Abstract of the Thesis of

Tara Joy Massad for the degree of Bachelor of Science

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Zebrafish neurogenesis is based on the complexity of molecular pathways and the simplicity of evolutionary conservation. Cells in the developing ectoderm must "decide" to become neural cells or epidermis and later whether to differentiate early into primary neurons or into later-developing neural cell types. This work examines two developmental mechanisms; the first acts through diffusible signals, setting up medioloateral boundaries within the developing neural ectoderm using the proteins BMP-4 and Shh. The second, the lateral inhibiton pathway, functions between neighboring cells, directing differentiation between primary sensory neurons and later-developing neural

crest. Studying mutations that alter these mechanisms served as a powerful tool for determining the actions of the developmental signals in wild-type embryos. Results from the first experiment suggest the absence of midline signals does not greatly alter patterning in the dorsal neural tube. The second study suggests the *mind bomb* gene, as defined by the *mind bomb* mutation, lies upstream of a ligand in the lateral inhibition pathway.

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INTRODUCTION

Developmental biology addresses a fascinating scientific question—how a single cell becomes a multicellular organism as complex as a nematode, a fish, or even a human. For a process reliable enough to create generation after generation of organisms with forms that remain true to the specific pattern of their species, the system is both amazingly complex and resourceful. In the text, *Developmental Biology*, Scott Gilbert summarizes this intricacy, writing that development "generates cellular diversity and order within each generation, and it assures the continuity of life from one generation to the next."[1] Yet, even with as many detailed signaling pathways as there are controlling the fate of each cell in a body, evolution has preserved many of the mechanisms so that, by studying fruit fly development, one is also learning much about his/her own embryonic history.

For my thesis, I have worked in Dr. Judith Eisen's developmental neurobiology lab, focusing on the processes by which specific types of neural cells arise in predictable positions along the spinal cord of *Danio rerio*, the zebrafish. Studies that focus on neural development have value beyond any direct discoveries made because knowledge gained has the potential to illuminate the details underlying diseases of the nervous system and to assist in the creation of therapies to counteract them. Because the development of the nervous system in zebrafish, like many developmental processes, has been highly conserved throughout evolution, valid extrapolations about human development can be made based on its exploration. Many analogous genes and, therefore, proteins are found in organisms ranging from *Drosophila melanogaster*, the fruit fly, to *Xenopus laevis*, the African clawed frog, to the zebrafish which are the subjects of these studies.

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[1] Gilbert, 1988, p.4

The induction of two of the earliest developing cell types in the nervous system, primary sensory neurons and neural crest, occurs very early in zebrafish (at about nine to ten hours post fertilization or at the 90 percent epiboly to bud stage) (Appel and Eisen, 1998). My research has focused on steps involved in the differentiation of these cells. By studying embryos with mutations in early nervous system development, much can be learned about the processes involved in cells committing to different fates and the signals governing such events.

I have conducted experiments dealing with the patterning of neural crest and primary sensory neurons. In the first set of experiments, I looked at the effects of diffusible signals that originate from midline tissue rather than within the nervous system. I compared embryos with mutations that exhibit a decrease in these signals, *floating head,* and embryos mutant for two such genes, *cyclops* and *floating head*, with wild-type embryos in an attempt to discern any differences in neural crest expression when signals involved in establishing ventral nervous system cell fates are reduced as is the case in the mutants. The second group of experiments focused on a local signaling pathway that acts between neighboring cells of the nervous system. The process, known as lateral inhibition, inhibits the development of primary sensory neurons from undifferentiated cells that, without the signaling process, will take on this early neuron fate. If their differentiation is blocked by this mechanism, it appears that they will remain undetermined for a time and then become neural crest. The zebrafish mutant, *mind bomb (mib)*, has an overabundance of primary sensory neurons at the expense of neural crest, and therefore seems to have a mutation in this pathway. I hoped to identify which piece of the signaling process is defective through injecting embryos with several mRNAs encoding for proteins in the pathway (mRNA is the RNA which encodes for protein

synthesis). I looked for results showing the mRNA has the capability to rescue the mutant to the wild-type phenotype.

BACKGROUND

Development is based on two key steps that direct cell fate: determination and differentiation. Determination is the process in which a cell becomes committed at the molecular level to adopting a certain identity, and differentiation, the physical change of a generic cell into a specialized one, follows (Gilbert, 1988, p. 246). In a sense development truly is that basic—certain rules and processes are found governing cell fate decisions in all organisms thus far studied, suggesting the mechanisms have been conserved throughout evolution. The complexity and specificity of the "art" is found in the numerous pathways and signaling mechanisms underlying these changes. All organisms start as a single cell, but how does this one cell—this one nucleus, one set of chromosomes—become a complex, multicellular creature with such a diversity of cell types? It is this question that developmental biologists attempt to answer, piece by piece resolving the systems governing gene transcription and how this regulates cell differentiation.

Along the dorsal region of vertebrate embryos, the developing nervous system consists of a flat neural plate which folds up over itself and forms the neural tube as development proceeds (See Figure 1). The neural plate contacts an underlying region of mesoderm cells called the notochord, a signaling center important for development. These cells induce the floor plate, a group of specialized cells found at the medial midline of the neural plate and also necessary as a signaling center (See Figure 2). Neural crest cells form at the lateral edges of the neural plate and become the dorsal-most cells in the nervous system as the tube closes. These cells then migrate out from the dorsal region and continue to differentiate into many cell types including pigment cells, neurons, and glia (cells that support neurons) of the peripheral nervous system. The fates of these lateral neural plate cells are

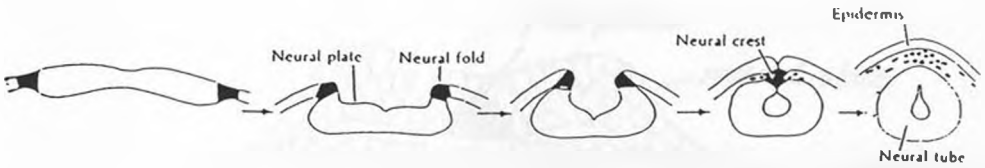


FIGURE 1a. This diagram shows the formation of the neural tube as the neural plate, located dorsally (on the back side of the embryo), folds up over itself. The black areas on the edge of the neural plate become the neural crest cells. These cells can then be seen migrating out laterally where they will later form pigment cells and peripheral neurons (reproduced without permission from Gilbert, 1988, p.155).

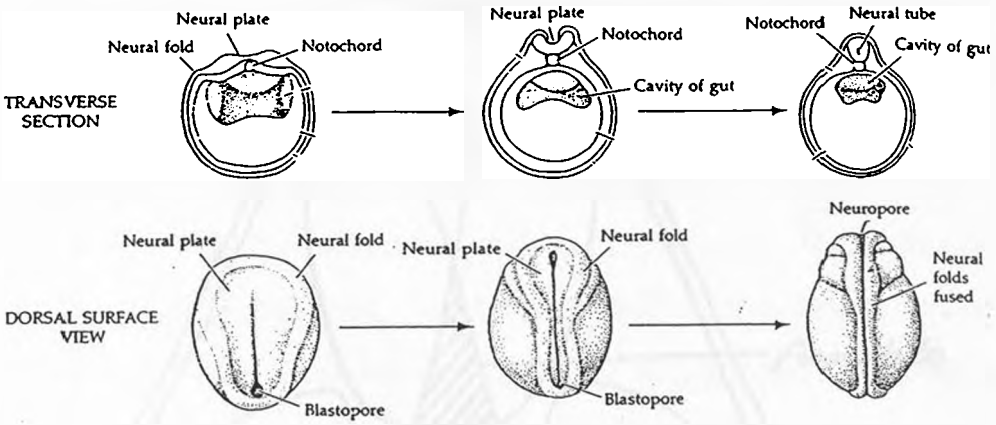


FIGURE 1b. This figure illustrates the same process through a cross section of the embryo and also with a dorsal view of the entire embryo (reproduced without permission from Gilbert, 1988, p. 156).

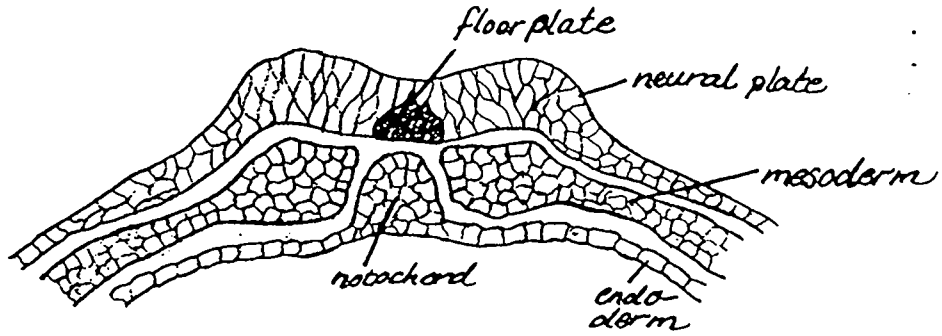


FIGURE 2a. This illustration is a diagram of the neural plate underlain by the floor plate and notochord.

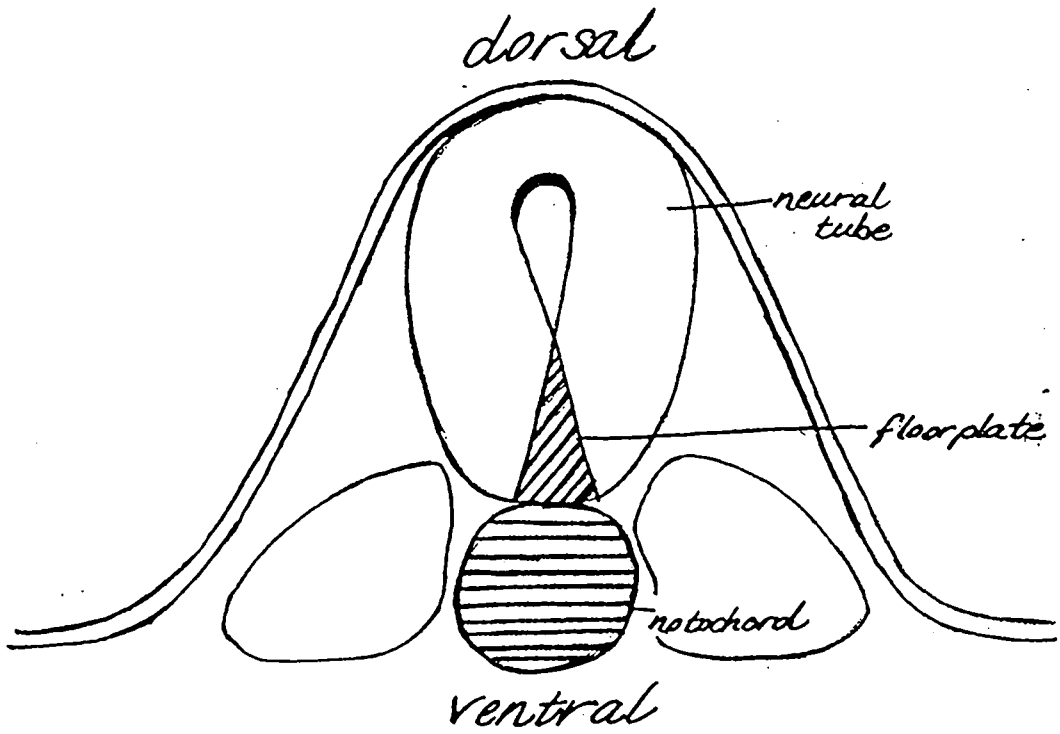


FIGURE 2b. This shows the position of the floor plate and notochord after the neural plate has closed into the neural tube.

thought to be controlled by two separate proteins, Bone morphogenic protein-4 (BMP-4) and Sonic hedgehog (Shh).

BMP-4 acts in a graded fashion with its highest concentration in the epidermal ectoderm (found adjacent to the neural plate at this time; later covers the neural tube dorsally) (reviewed in Mayor *et al.,* 1999). In vitro it has been shown to induce neural crest in neural plate explants. The same explant work has shown the protein, Shh, blocks neural crest induction (Liem *et al.,* 1995). In vivo, Shh diffuses from the notochord and floor plate. BMP-4 is suspected of dorsalizing the neural plate locally in vivo, diffusing toward the medial neural plate and, at middle concentrations, inducing neural crest (review in Mayor *et al.,* 1999). Shh, on the other hand, seems to create a ventral boundary in the neural tube for neural crest cells. These results were found with in vitro experiments using chick tissue (Liem *et al.,* 1995) which, in summary, illustrate that the epidermal ectoderm contains the dorsal signal, BMP-4, which diffuses and can oppose the ventral, notochord signals and encourage cells to adopt dorsal fates (ie, neural crest). Chick cells exposed to the ventral signal, Shh, however, do not adopt dorsal neural crest fates, showing that notochord-derived signals have the potential to depress neural crest development (Liem *et al.*, 1995). Therefore, it seems likely that zebrafish mutants lacking notochord and/or floor plate will have a ventrally expanded response in vivo to BMP-4 signals since Shh is absent to counteract them. *floating head (flh)* mutants and *cyclops/floating head (cyc; flh)* double mutant embryos were examined to determine if this is truly the case. *flh* mutants lack notochord and *cyc; flh* mutants are missing both floor plate and notochord. I performed RNA in situ hybridizations on 24 hour embryos using the dorsal neural tube markers *msxb, apa2, pax3, pax7* and *gli.* I hypothsized these markers would be expanded ventrally, indicating a neural crest expansion. The

intermediate neural tube marker, *pax2*, was also examined in order to see if Shh perhaps would have greater effects in intermediate, rather than dorsal neural tube.

The second set of experiments focused on the highly conserved Delta-Notch lateral inhibition signaling pathway, so called because unlike the interactions of the diffusible BMP-4 and Shh proteins, it functions to inhibit equivalent development in adjacent cells (Artavanis-Tsakonas *et al.*, 1995). This pathway is responsible for multiple developmental processes in differing organisms, but in all cases thus far examined, proteins of the Delta/Notch signaling cascade are involved. Delta is a membrane bound ligand with four homologues in the zebrafish, DeltaA, DeltaB, DeltaC, and DeltaD (Dornseifer *et al.*, 1997; Haddon *et al.*, 1998; Appel and Eisen, 1998). It functions by binding the Notch transmembrane receptor which, when bound, releases its intracellular domain (N^{ICD}). This piece of the protein binds to the Suppressor of Hairless (Su(H)) protein; it is this complex of N^{ICD} and Su(H) which enters the nucleus and acts as a transcription factor, binding the cell's DNA and activating the transcription of genes within the Enhancer of Split (E-spl) DNA complex (reviewed in Beatus and Lendahl, 1998) (See Figure 3). Proteins encoded by these genes are responsible for the repression of the primary sensory neuron fate (Wettstein *et al.*, 1997). It then follows that cells with Delta bound to them will not be able to become primary sensory neurons and will instead adopt other fates which are induced later in development, such as neural crest (Cornell and Eisen, 1999, submitted).

Cells in the neural plate initially all express both Delta and Notch, but the pathway is regulated through a feedback loop, so when a cell experiences a high concentration of bound Delta, the Notch receptor initiates a chain of events resulting in the repression of transcription of the *delta* gene. If, however, a cell's Notch is not

The Delta-Notch Lateral Inhibition Signaling Pathway

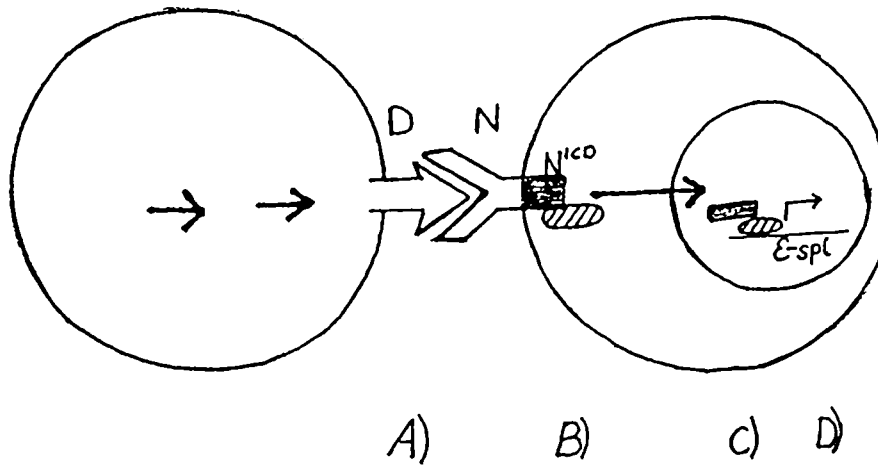


FIGURE 3. This is an illustration of the Delta-Notch lateral inhibition signaling pathway acting in two neighboring cells. This shows the generalized form of the pathway. I have described it specifically for zebrafish primary neuron differentiation, although this is the process I am trying to test.

A) Delta is a protein signal held in the cellular membrane of the first cell. It binds the Notch protein receptor which is located in the cellular membrane of the second, neighboring cell.
B) The intracellular domain of Notch (N^{ICD}) is bound to the Suppressor of Hairless (Su(H)) transcription factor (a protein that initiates DNA transcription).
C) Once Notch is bound by Delta, the N^{ICD} and Su(H) break loose from the rest of the Notch protein and enter the cell's nucleus.
D) This complex of N^{ICD} and Su(H) is then able to activate transcription of the *enhancer of split* (E-spl) genes which results in the prevention of the cell from adopting a primary neuron fate.

activated, *delta* transcription will increase and Notch transcription will be relatively lower (reviewed in Artavanis-Tsakonas *et al.*, 1995). Through these regulatory mechanisms, cells, which have the potential to express both Delta and Notch, are limited to adopt one specific fate.

RNA In situ hybridizations show that zebrafish *delta* genes are expressed beginning at 90 percent epiboly in the epiblast (future neural ectoderm) (Haddon *et al.*, 1998). Injecting *deltaA* or *deltaD* mRNA into zebrafish results in the translation of the injected RNA into proteins and the subsequent inhibition of neurogenesis, judged by decreased expression of *islet1*, a marker for early-developing neurons. Similar, but weaker, effects are seen with *deltaB* injections (Haddon *et al.,* 1998). Injections of RNA encoding *Xenopus* dominant negative *delta* have the opposite effect, resulting in an increased expression of *islet1* (Haddon *et al.,* 1998). Dominant negative mutations are those that counteract wild-type phenotypes because they are dominant to the wild-type allele of the gene and interfere with the function of wild-type proteins. These results support the mechanism of the Delta-Notch pathway outlined above. The conclusions also demonstrate a function that is preserved across species because similar results are found in tests with *D. melanogaster* and *X. laevis* (Chitnis *et al.*, 1995).

Mutants in the Delta-Notch pathway with damaged Delta, Notch, or Su(H) are likely to show a phenotype similar to that seen in embryos injected with the RNA encoding the dominant negative form of *delta*. Therefore, an increase in primary neurons, such as the Rohon Beard (RB) primary sensory neurons would be predicted. The zebrafish mutant, *mind bomb (mib)*, shows such a phenotype with an overexpression RBs and a decrease in neural crest (Jiang *et al.*, 1996). This suggests that cells in the lateral neural plate (dorsal spinal cord) can become either RBs or neural crest, but the preferred fate is RBs and that because Delta signaling

normally prevents most cells from becoming RBs, they become neural crest. It is therefore reasonable to hypothesize the *mib* mutation is due to a mutation affecting a protein in the Delta-Notch pathway. In this way, the system may bottleneck due to the malfunctioning of just one link in the chain of molecular events in this pathway. In an attempt to answer these questions, I performed experiments to determine if *mib* can be rescued with the injection of mRNA encoding for different proteins in the Delta-Notch pathway.

MATERIALS AND METHODS

Zebrafish Embryos

Zebrafish raised in the University of Oregon Zebrafish Facility were used for the experiments. The fish were raised at 28.5^0 C and staged using Kimmel *et al.* (1995) as a guide. Crosses of heterozygous *cyc; flh* and *mib* mutants were performed to obtain homozygous mutant embryos. I raised *cyc; flh* embryos at 28.5^0 C or 32^0 C for 24 hours. I then fixed them in four percent paraformaldehyde at 4^0 C overnight. I raised *mib* embryos at 32^0 C until the six to eight somite stage. At this point, I fixed them in two percent paraformaldehyde at 4^0 C overnight.

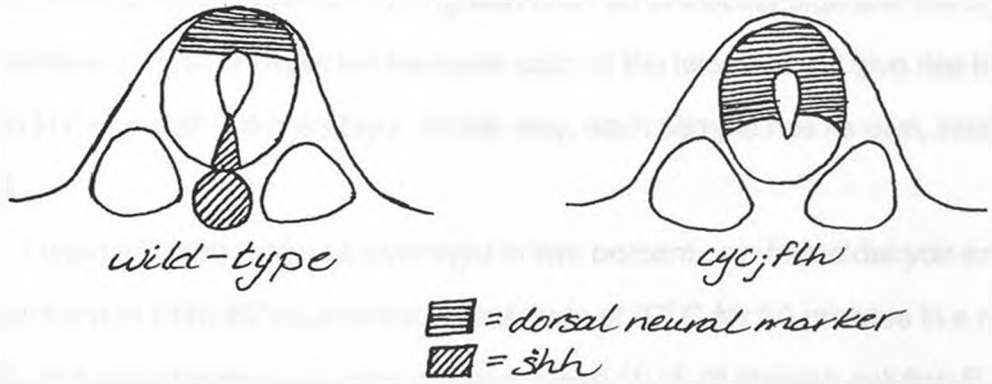
Probe Synthesis and RNA In Situ Hybridization

RNA in situ hybridization is a staining process that labels cells which naturally express an RNA that becomes bound by an anti-sense RNA probe. The probes are labeled with digoxigenin and, when added to embryos, will bind the cells' own RNA. After hybridizing the probes to the embryos' own RNA, I washed the probe from the embryos and added a solution with anti-digoxigenin secondary antibody. The antibody binds the digoxigenin labels on the probe. Finally, I developed the RNA in situs using two chemicals, NBT and BCIP, which act on the antibody and leave a blue reaction product, thereby staining the cells with the probe bound blue. This enables one to see the natural expression patterns of RNA in order to identify cell types. Methods of probe synthesis and in situ hybridizations used were those standard to the Eisen lab as described in the manufacturer's instructions (Boehringer Mannheim) and Thisse *et al.* (1993) respectively.

Scoring *flh* and *cyc; flh* Mutant Embryos for a Ventral Shift in Neural Markers

I examined embryos both as whole mounts and in cross sections after they were stained by RNA in situ hybridization. I looked in their spinal cords for a ventral

shift in neural markers. Therefore, I was concerned only with vertical changes in marker expression and not lateral effects as these may be due to neural crest cell migrations. (See Diagram).



RNA Synthesis and Injections

I transtribed RNA from purified plasmid DNA using the Ambion SP6 Message Machine Kit and the instructions specific to it. I performed injections with *slz* mRNA (which encodes ß-galactosidase) and either N^{ICD}, *Xenopus delta-1*, or zebrafish *delta A* mRNA, in a 1:1 mixture. I added a small amount of phenol red dye to the RNA, so it could be seen during the injections. I injected only one cell of a two-cell embryo under a microscope with fine needles made from capillary tubes. I then raised all embryos in embryo medium with penicillin streptomycin (an antibiotic) at pH 7.3. I also grew those injected with N^{ICD} in a solution of 20 µM dexamethasone made from a stock solution of 20,000 µM dexamethasone in DMSO. Dexamethasone is a steroid hormone which assists with the intake of the N^{ICD}-Su(H) complex to the nucleus.

Development of ß-galactosidase Stain and In Situ Hybridization

The *slz* mRNA injected encodes for the enzyme ß-galactosidase which when developed with the substrate, X-gal (5-bromo-4-chloroindole), for one half hour at 37^0 C cleaves the galactose from the X-gal, leaving behind a blue precipitate as the

reaction product (Gilbert, 1997, p. 56). This functions as a lineage tracer for the injected RNA because the cells stained blue are those containing the injected RNA. Because I injected only one of the two cells in the embryo, an ideal result is an embryo which shows unilateral staining with one half of it being blue and the other half unaffected. This is expected because each of the two cells will give rise to the cells on just one half of the embryo. In this way, each sample has its own, internal control.

I fixed injected embryos overnight in two percent paraformaldehyde and then washed them in PBS-NP40 and incubated them at 37^0 C for 30 minutes in a mixture of 1 mL of ß-galactosidase staining solution A and 10 µL of staining solution B which contains X-gal. I then washed them in PBS-NP40 and re-fixed them in four percent paraformaldehyde overnight at 4^0 C. After fixing, I washed the embryos again, dechorionated them (removal of the outer, protective chorion layer), and developed them using the standard RNA in situ procedure described above.

RESULTS

Loss of Floor Plate and Notochord Does Not Affect Neural Crest Patterning

I fixed wild-type, *flh,* and *cyc; flh* embryos at 24 hours and stained them using RNA in situ hybridization for one of six different neural crest markers and the ventral signal, Shh (See Figure 4). None of the dorsal markers, *ap2, gli, msxb, pax3,* or *pax7,* showed convincing ventral expansion in embryos lacking floorplate or notochord. *pax2* is naturally the most medially expressed of the markers, and it was the only one to display a ventral shift from its natural intermediate position. I examined the embryos both as whole mounts and in cross section, but, with the exception of *pax2*, no changes in marker expression were present in either. Wild-type embryos had Shh expression in both the floor plate and notochord region, and *flh* mutant embryos showed random patches of expression in "islands" of floor plate that remain in the mutants (Halpern *et al.,* 1995). *cyc; flh* mutants were completely without Shh expression at the age examined. In all these cases, however, expression of the dorsal markers, *ap2, gli, msxb, pax3,* and *pax7,* appeared like that seen in wild-types. This was an unexpected result based on the presumption zebrafish embryos would behave as did the chick tissue in Liem's work (Liem *et al.*, 1995).

Zebrafish experience a dose of early Shh activity during gastrulation, and it may be this exposure that sets up the dorsoventral neural patterning (Beattie *et al.,* 1997). Therefore, a lack of Shh later in development would not have an effect in neural crest induction although it is responsible for it at this earlier point. To test this model, I performed RNA in situs using the *msxb, pax2, pax3*, and *pax7* markers on a third mutant, *b641.* I also used another intermediate neural tube marker, *pax6.* This mutant seems to lack the Smoothened receptor for Shh, so no Shh activity can

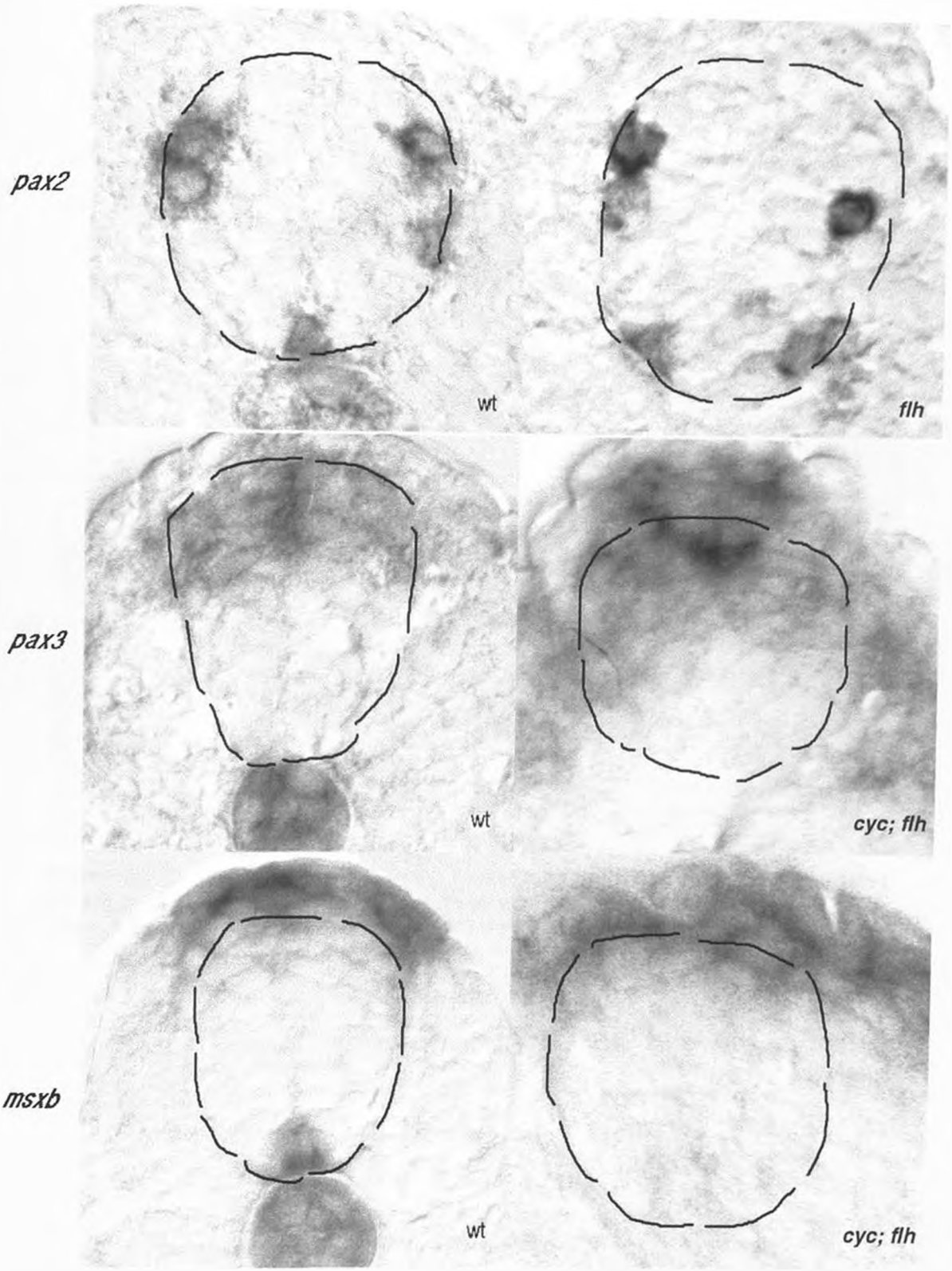


Figure 4a. Cross sections through the neural tube of RNA in situs for *shh* and and neural markers on 24 h wild-type and *flh* or *cyc; flh* mutant embryos. *pax2* is the only marker with a ventral shift in the spinal cord in mutants lacking notochord and floorplate. The other markers appear unaffected by the mutations. The outlined areas are the spinal cords.

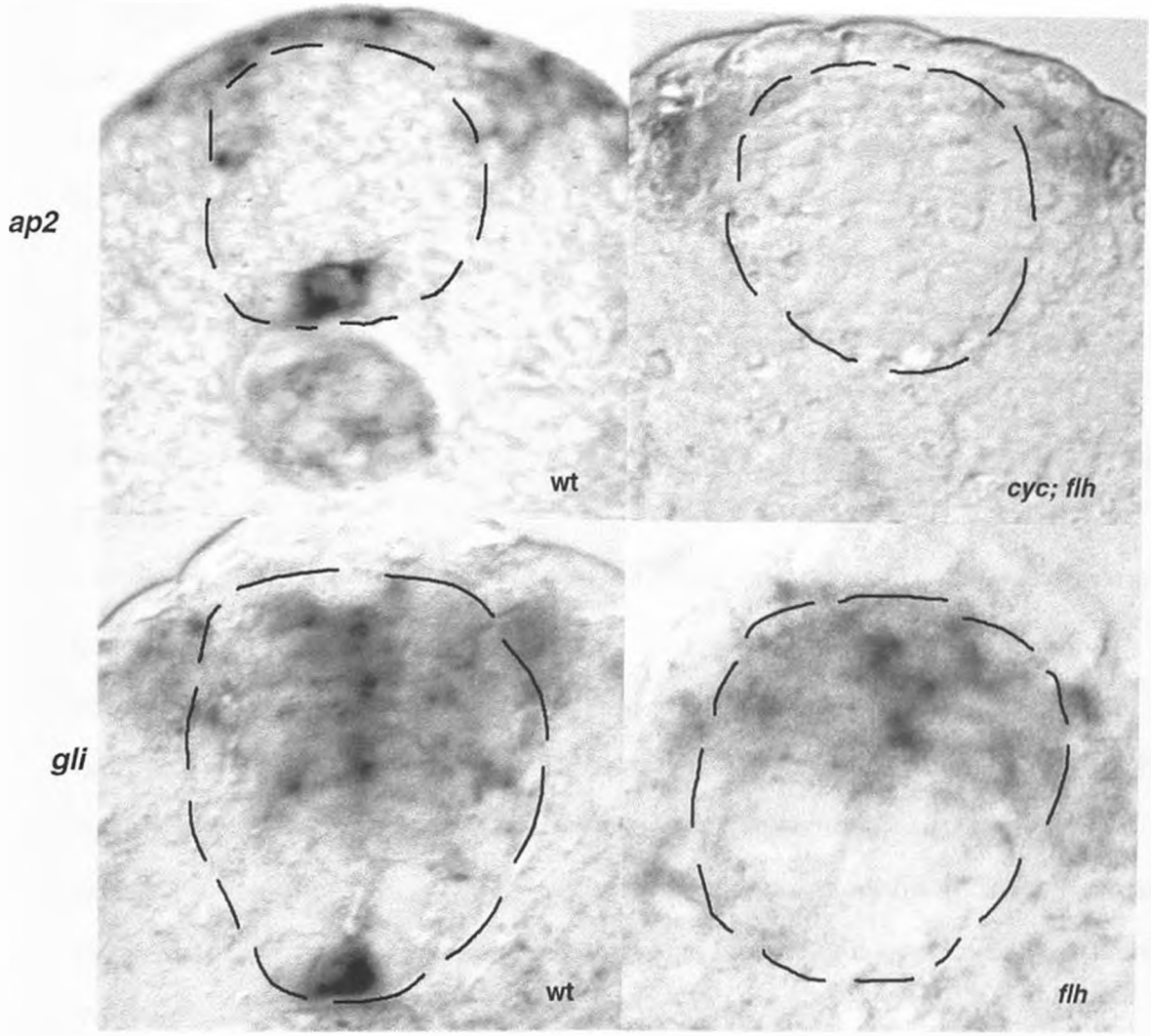


Figure 4b. Cross sections through the neural tube of RNA in situs for *shh* and and neural markers on 24 h wild-type and *flh* or *cyc; flh* mutant embryos. No ventral shift is seen in the markers in mutant embryos. The outlined areas are the spinal cords.

ever be detected by the mutant, providing a closer simulation of a complete knock-out of the protein (Varga and Westerfield, 1999, personal communication). Results, however, closely mimic those found in the *flh* and *cyc; flh* mutant embryos. Yet, *pax2,* did not show the ventral shift found with the other mutants where as *pax6* and *pax7* did show a ventral shift in the spinal cord (data not shown). They too, are naturally more intermediately expressed than the other markers.

mib Mutants Can Be Rescued with _Xenopus delta-1_ and Activated _notch_ mRNAs

I injected both *mib* mutant and wild-type embryos with mRNA encoding ß-galactosidase and either the N^{ICD}, *Xenopus delta-1* or the zebrafish *deltaA*. I performed the injections at the two cell stage and injected only one cell of each embryo. This allowed for an internal control in which the experimental side and the control were in the same embryo. Wild-type fish injected with these mRNAs showed a decrease in the development of primary sensory neurons as expected based on the known function of these genes in mediating lateral inhibition (reviewed in Beatus and Lendahl, 1998; Haddon *et al.*, 1998).

In addition, I found that injecting *mib* mutant embryos with N^{ICD} mRNA resulted in a reduced number of primary sensory neurons (See Figure 5). This leads me to the hypothesis that the mutation is either in *notch* or in an earlier step in the pathway. To further isolate the mutation, I injected *mib* mutants with mRNA encoding Delta to determine if the mutation lies in *notch* or upstream of it. *Xenopus delta-1* mRNA, which is known to block neurogenesis in zebrafish, encodes a form of Delta that functions in place of all the zebrafish Delta homologues, so I tested its effects first. It too rescued the mutant phenotype, resulting in a reduction of RB cells (See Figure 5). Of the 94 wild-type embryos that were injected, 93 showed a reduction in primary neurogenesis. Of 11 *mib* mutant embryos I injected, nine

exhibited a strong reduction in RB cells and four were slightly affected. This led me to hypothesize that *mib* must be in *delta* or in a gene upstream of it, for example something involved in processing Delta or getting it to the cell membrane.

To narrow down the possible locations of the *mib* even more, I injected an RNA encoding a more specific *delta*, zebrafish *deltaA* into both wild-type and *mib* embryos. However, results with this construct were never as strong as those found with the *Xenopus* RNA, and, though it reduced primary neurogenesis in wild-types, I observed only a slight reduction in RB cells in *mib* mutants (See Figure 5). Titration experiments with *deltaA* mRNA injections at half and one-third strength concentrations were performed to test quantitative effects of differing DeltaA levels. The low dose of *deltaA* mRNA did not affect the number of RB cells in either wild-types or *mib* mutants (data not shown). Therefore, the cut-off level for functional amounts of *deltaA* is the same within a narrow range for both wild-type and the mutant.

Finally, to test the timing of *delta* expression in *mib* mutants as compared to wild-type, I performed RNA in situs with a *deltaA* (the earliest of the four *deltas* to be transcribed (Haddon *et al.*, 1998)) probe. Haddon looked at expression of zebrafish *deltaA, deltaB*, and *deltaD* in the ear of *mib* mutants, but her study used embryos at 14 and 18 hours post fertilization (Haddon *et al.*, 1998). Zebrafish neurogenesis is in motion much earlier, starting during gastrulation, at about five hours post fertilization (Gilbert,1988, p. 155; Westerfield, 1994, p. 3.57). Therefore, I performed this experiment on a series of embryos at shield stage (six hours), 75 percent epiboly (eight hours), 90 percent epiboly (nine hours), and bud stage (10 hours). I examined the embryos for the pattern and concentration of staining to see if perhaps *mib* mutants do not express *deltaA* as early as wild-type embryos, thereby suggesting a reason behind the increase in primary neurogenesis. Results,

however, were the same for all embryos as staining among those in each age group was consistent (data not shown).

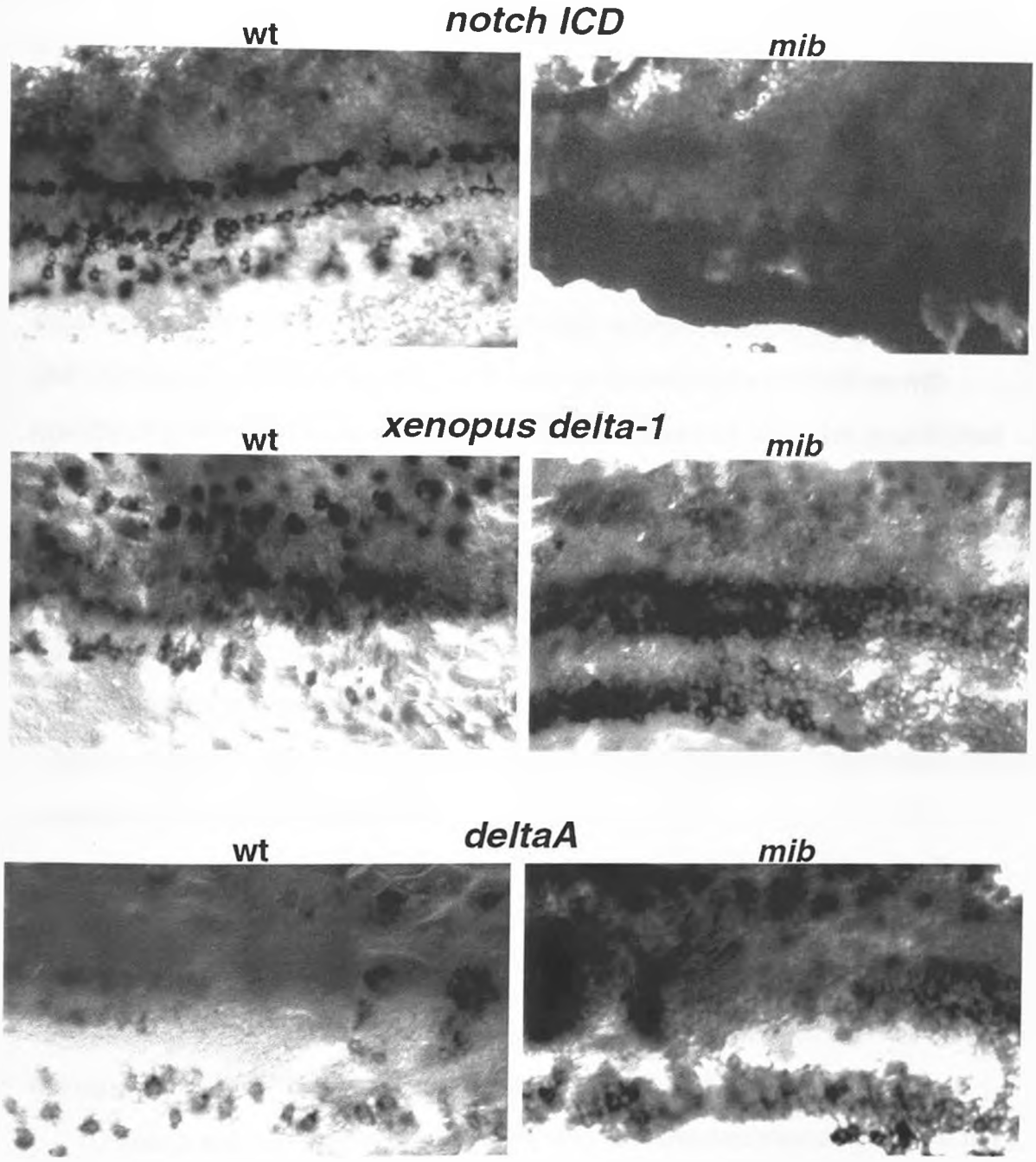


Figure 5. *mib* injections. Dorsal views of unilaterally injected embryos show a reduction of Rohon Beard cells (purple stained cells) in the experimental half (blue stained region) of the embryos.

DISCUSSION

Effects of Midline Mutations on Neural Tube Patterning

The results of the first set of experiments involving RNA in situs with *flh* and *cyc; flh* mutants were contrary to the original hypothesis that zebrafish lacking floor plate and notochord, and therefore a ventral source of Shh and other midline signals (Beattie *et al.*, 1997), would have developmental irregularities similar to results from chick explant experiments. These chick in vitro experiments show that not only does BMP-4 induce dorsal neural tube cell fates, but that cells grown in culture with notochord give rise to reduced expression of *PAX3* and *DLS1* (chick neural crest markers) (Liem *et al.*, 1995). This led to the expectation that reduced Shh in zebrafish would result in an expansion of dorsal neural tube markers. Yet, in *flh* and *cyc; flh* mutant embryos, which are missing midline structures and therefore the normal source of Shh, the only marker with any change was *pax2*, which is an intermediate, not dorsal marker, and appears ventralized. This suggests the inhibitory factors from the midline may reach only to the middle of the embryo rather than all the way to the dorsal side.

It is hypothesized that BMP proteins work in a graded fashion, specifying neural plate, neural crest and nonneural cells in order from lowest to highest concentration (reviewed in Mayor *et al.*, 1999). It is then also possible that ventralization does not occur because the necessary dose of BMP is not present to induce an exaggerated neural crest regardless of Shh activity.

These are two possible explanations for the discrepancy observed in the development of chicks and zebrafish. Aside from prematurely deducing that zebrafish and chicks must simply develop differently, there are also other conclusions to weigh as well. It may be that the signals are working in the same way, but what is found in vitro does not hold true in vivo. There is a definite difference

between the power of in vitro and in vivo experiments as the former can only illustrate what may be the case within developing cells and the latter shows how the actual mechanisms at work in the growing organism function. Because of this, zebrafish are especially valuable to work with since, unlike chicks, mutants exist that can be used to discern what goes on in vivo when the midline signals are absent, and this may be significantly different from what happens when explants of tissue are cultured. This could mean the two organisms do not develop differently at all, and that, though Shh may have the potential to limit dorsal genes, it does not actually fulfill that function in nature. So, as the Liem experiments show, Shh does inhibit neural crest fates, but as our zebrafish work suggests, Shh is not working this way at this stage of in vivo development. Relating to the gradient model, perhaps BMP-4, the dorsal fate signal, is not active ventrally enough to induce cells to adopt such identities. Therefore, Shh activity would be irrelevant since ventral cells would not sense BMP-4.

Another explanation may be found in the role of a peak of Shh at the end of zebrafish gastrulation which is still present in *flh* and *cyc; flh* mutants (Beattie *et al.*, 1997). It may be that this early expression is responsible for the ventral restriction even later in development because primary motoneurons are actually specified at the end of gastrulation, at which time their precursor cells are next to the axial mesoderm where *shh* is active (Beattie *et al.*, 1997). I did further experiments with this mechanism using another mutant called *b641* which shed more light on this possibility that timing is different in zebrafish and chick Shh activity. Based on genetic mapping data, this strain of fish appears to lack Smoothened, part of the receptor for Shh (Varga and Westerfield, 1999, personal communication), creating an effect closer to a complete loss of Shh because, regardless of Shh levels, none can be effectively detected by the mutant. I performed the same series of RNA in

situs and an RNA in situ with the additional intermediate neural tube marker, *pax6,* with this mutant in hopes of clarifying the role of Shh in dorsal neural patterning. Because results in the mutant appear identical to those in wild-type embryos for dorsal markers, it seems even this lack of early Shh activity and inability to sense Shh at any time does not affect ventral boundaries within the developing nervous system.

In summary, this work suggests that in the absence of midline signals, dorsal cells develop normally and the dorsal fate does not expand ventrally. So, though such signals may have the potential to affect ventralization, they do not seem to play this role in a natural, in vivo setting. Yet, the possibility that changes in dorsoventral patterning did occur, but were too subtle to be detected does remain, as does the chance this mechanism may be functional in zebrafish but only as a backup system that is able to direct patterning if the animal is experiencing stress during development.

Possible Locations of the *mib* mutation in the Delta-Notch Lateral Inhibition Signaling Pathway

The second experiments also produced surprising results based on the original assumptions. The fact that *mib* mutants show such an abundance of early developing sensory neurons makes it seem as though the Delta-Notch lateral inhibition pathway that normally controls their proliferation is disrupted. Its phenotype is also very similar, although showing a more pronounced mutation, to the dlA^{dx2} mutant phenotype which has a mutation in *deltaA* (Appel *et al.*, 1999). The *mib* phenotype is also akin to the phenotype induced in wild-type embryos upon injection with mRNA encoding dominant negative X-Delta-1(Appel *et al.*, 1999). Therefore, I expected that a protein involved in the Delta-Notch lateral inhibition pathway would be mutated in *mib* mutants. The results from the RNA

injections all support this hypothesis as both the N^{ICD} and *delta* mRNAs were able to rescue the mutant, reducing the proliferation of Rohon Beard cells. This provides evidence that the *mib* mutation lies at the level of *delta* or further upstream still.

Yet, I first assumed *mib* would be downstream of Delta, the ligand (binding protein) that initiates the signal. Rescue results with *delta* mRNA were unanticipated for several reasons. First, since zebrafish have four similar Delta proteins, it is difficult to imagine that a mutation in one of the Delta homologues could be responsible for the *mib* mutant phenotype. The phenotype is so severely mutated that having a mistake in one of four related proteins would not be presumed to have such an effect. Even if one were mutated, only a slight change in phenotype would be expected since three other proteins would be available to compensate for the loss due to the mutation. Therefore, it seemed most likely that any mutation in this pathway would lie in a protein represented by only one homologue in zebrafish. Another argument against *delta* being the site of the *mib* mutation is the data from RNA in situ hybridizations that *mib* mutants, rather than lacking *delta* mRNA, actually have an abundance of it present (Haddon *et al.*, 1998). It seems strange, then, to think the *mib* mutation would be found in a *delta* gene because they appear to be expressed strongly. However, timing is always a critical issue in development, and it may be that expression of *delta* is so high because its early activity is missing. Since the lateral inhibition pathway regulates itself through a negative feedback loop, if Delta is not felt early on, it will not dampen its own activity in other cells, so more of them will express it. Because this same early Delta is responsible for directing sensory neuron differentiation, a mutation at this point would result in an excess of both primary sensory neurons and relatively late-acting Delta. The results of the RNA in situ work showing the timing of *deltaA* RNA is consistent in *mib* mutant and

wild-type embryos call into question this idea, yet the differences in timing may be as little as a few minutes and therefore were not detected. Furthermore, the in situs detected only RNA levels and not those of the protein, so it may be that transcription is timed normally, but translation is delayed. Also speaking against the *mib* mutation being in *delta* is unpublished chromosome mapping data which shows *delta* is not the site of the *mib* mutation (Eisen, 1999, personal communication). Furthermore, genetic complementation tests have shown the *mib* mutant and the $dlA^{\phi x2}$ mutant do not complement, showing *mib* is a mutation in a different gene than *deltaA* (Appel *et al.,* 1999).

The first injections of N^{ICD} resulted in a positive rescue, showing the mutation did lie in the Delta-Notch signaling pathway. It pointed to the mutation being in Notch itself or perhaps in a protein preceding it. The follow-up experiments attempted to further isolate the mutation by examining the effects of having functional Delta present. I first injected *Xenopus delta-1* because it is a general form of Delta that had previously been shown to work in zebrafish (Haddon *et al.,* 1998). Injections of the *Xenopus delta-1* mRNA rescued *mib* mutants, based on data showing a reduction in primary sensory neurons. Because *mib* has been shown by others not to be a mutation linked to any zebrafish Notch or Delta (Eisen, 1999, personal communication), this suggests that *mib* is upstream of Delta in the pathway. In hopes of pinpointing the mutation more specifically, I followed this experiment with injections of zebrafish *deltaA* mRNA. However, I never attained a complete rescue with this construct. Perhaps this is because *deltaA* works fine in *mib* mutants, and a protein affecting one of the other homologues is mutated. It is also possible that *Xenopus delta-1* works simply because it is a more general form of *delta* that effectively saturates the developing dorsal nervous system with ligand.

All data taken together, it seems most likely *mib* is a mutation of a gene that affects the lateral inhibition pathway somewhere upstream of Delta. The puzzle of where *mib* lies is truly complicated, and the severity of the mutation makes quantitative explanations that suggest only a decrease in protein levels, rather than a complete knock-out, unlikely. Such a gene may encode a protein involved in the processing of Delta protein. For example, there is a protein called Kuzbanian that is known to cleave Delta, thereby promoting its activity (Artavanis-Tsakonas *et al.*, 1999). If such a protein is operative only at low levels due to a mutation, it may not be able to produce a functional quantity of Delta in *mib* mutants. However, I performed titration experiments with *deltaA* mRNA injections at differing concentrations to test this quantitative model. Since the cut-off level for functional amounts of *deltaA* was shown to be the same within a narrow range for both wild-type and the mutant, it seems as though any proteins affecting *delta* activity are also present at equal levels.

Because *mib* mutants have high expression of *delta* RNAs, the mutation seems to affect a step in the pathway after the level of delta transcription, and because *mib* mutants can be rescued with *delta* mRNA, it seems these mutants must be capable of translating the mRNA into functional protein and processing it as normal. This complicates possible hypotheses for *mib's* location even more by limiting hypotheses suggesting *mib* is a mutation in the translation machinery of *delta*. Yet, it may be the mRNA produced naturally in *mib* mutants really is not translatable and because the injected mRNA has a 5' methyl capped guanosine, which promotes translation, it is able to function. The timing model may also be able to clear this up because if Delta is not transcribed or translated early enough, it will not be able to limit primary neurogenesis.

A final idea is that one or more of the proteins involved in Delta processing are responsible for localizing it on the cell surface, and that somehow they malfunction and Delta never arrives in its functional position. Simply stated, the mere fact so many hypotheses for the location of *mib* remain shows it is still quite a mystery, and much is left to discover with regard to the proteins that play a role in the lateral inhibition pathway.

CONCLUSION

These experiments have focused on two separate evolutionarily-conserved developmental mechanisms in zebrafish. We have looked at the effects of the diffusible signal, Shh, on the ventralization of the neural tube and concluded that though it has the potential to block dorsal signals and induce a ventral phenotype, it may not be fulfilling that role in vivo in zebrafish. It is interesting to see this because based on in vitro experiments, one may expect Shh to act as a ventralizing agent in vivo, but work with the *cyc* and *flh* mutants shows that in its absence, little is changed with respect to dorsoventral patterning in the zebrafish neural tube. This illustrates the important point that potential does not always equal function. It also further illustrates the power of genetic analysis through experiments involving mutants.

We have also examined the Delta-Notch lateral inhibition signaling pathway which is vital in directing differentiation between neighboring cells across genera. Looking at mutants is a valuable way to learn about the natural function of genes because one is able to see the effects produced without their normal expression. Therefore, it was worthwhile to study the *mib* mutant, which seemed to have a defect in the lateral inhibition pathway, in order to learn more about the proteins and processes involved in it. Our work helped narrow down the search for the malfunctioning gene in *mib*, pointing to it being it somewhere upstream of *delta* in this lateral inhibition.

Both these sets of experiments have focused on common proteins and mechanisms important to development. One of the most amazing aspects of this work comes from the evolutionary perspective in that functions of these proteins have quite often been conserved across species. This is an enormously valuable property because it enables specific research to be applied to multiple organisms. Such generalities spur researchers to hypothesize explanations for developmental

discrepancies that do not simply define them as innate differences between genera. Yet, subtle specificities do exist; therefore it is worthwhile to attempt to duplicate results between organisms in order to elucidate all the functions of different developomental regulators. For example, we found a discrepancy between the roles of Shh in chicks and zebrafish based on comparisons of in vitro and in vivo work, respectively. These "copy" experiments allow researchers to learn more about the multiple roles and times a regulator protein may act in different species.

Development is striking because of the dichotomy found in the similarity of its function and uniqueness of the outcome it facilitates across species. It is fascinating to discover the shared mechanisms by which all creatures grow and differentiate from a single cell, yet the importance of the work stretches farther than curiosity and mental challenge. Its practical value is in the enlightenment such studies can provide for researchers seeking to understand developmental disorders and diseases such as cancer. It is developmental data, for example, that can be used to learn how tumors grow and spread, and, through a familiarity with the genes involved in such processes, scientists arrive closer to preventing such conditions.

The work I have participated in is interesting, yet only a very small piece of the bigger story of how cells solve their "identity crisis" and adopt their final fates. It has addressed certain questions but has really done a more substantial job of creating more puzzles to ponder. Why is it that Shh is not working the same in chicks as in zebrafish? Is it merely an issue of timing or a real discrepancy between organisms? Will studies with the *b641* mutant be able to add to this knowledge as a better example of what happens if Shh is not detected? Where does the *mib* mutation actually lie? Furthermore, what other components make up the lateral inhibition pathway? Each experiment poses its own questions whose strongest purpose is perhaps to open the door to more inquiries and study.

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