Research article

Delta-Notch signaling and lateral inhibition in zebrafish spinal cord development

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Published: 16 July 2001

BMC Developmental Biology 2001, **1**:13

Received: 7 April 2001
Accepted: 16 July 2001

This article is available from: http://www.biomedcentral.com/1471-213X/1/13

Abstract

Background: Vertebrate neural development requires precise coordination of cell proliferation and cell specification to guide orderly transition of mitotically active precursor cells into different types of post-mitotic neurons and glia. Lateral inhibition, mediated by the Delta-Notch signaling pathway, may provide a mechanism to regulate proliferation and specification in the vertebrate nervous system. We examined *delta* and *notch* gene expression in zebrafish embryos and tested the role of lateral inhibition in spinal cord patterning by ablating cells and genetically disrupting Delta-Notch signaling.

Results: Zebrafish embryos express multiple *delta* and *notch* genes throughout the developing nervous system. All or most proliferative precursors appeared to express *notch* genes whereas subsets of precursors and post-mitotic neurons expressed *delta* genes. When we ablated identified primary motor neurons soon after they were born, they were replaced, indicating that specified neurons laterally inhibit neighboring precursors. Mutation of a *delta* gene caused precursor cells of the trunk neural tube to cease dividing prematurely and develop as neurons. Additionally, mutant embryos had excess early specified neurons, with fates appropriate for their normal positions within the neural tube, and a concomitant deficit of late specified cells.

Conclusions: Our results are consistent with the idea that zebrafish Delta proteins, expressed by newly specified neurons, promote Notch activity in neighboring precursors. This signaling is required to maintain a proliferative precursor population and generate late-born neurons and glia. Thus, Delta-Notch signaling may diversify vertebrate neural cell fates by coordinating cell cycle control and cell specification.

Background

Specification of cells at different times and places is critical for generation of cellular diversity in the vertebrate nervous system. Distinct types of neurons and glia develop at characteristic times and places [1] and molecular signals that promote formation of different cell types are regulated spatially and temporally [2]. Thus, neural pattern formation requires coordination of signals that provide spatial and temporal information.

Lateral inhibition, or lateral specification, is one process by which fine patterns of distinct cell types are generated

[3, 4]. Among cells that have the potential to adopt the same fate, lateral inhibition specifies some cells for a primary or preferred fate and others for a secondary or alternative fate. Cell ablation experiments are used to identify lateral inhibition: when cells that would normally adopt a primary fate are removed, cells that would otherwise acquire a secondary fate replace them. For example, when neuroblasts of the grasshopper central nervous system were ablated, they were replaced by cells normally fated to give rise to epidermis [5]. Similar demonstrations performed in other insects [6], leeches [7], nematodes [8], ascidians [9], and zebrafish neural crest [10] indicate that lateral inhibition operates in diverse developmental contexts throughout metazoan development.

The molecular mechanism of lateral inhibition is rooted in interaction of the ligand, Delta, and its receptor, Notch [11]. Various lines of evidence indicate that Delta, expressed by specified cells, activates Notch in neighboring cells directing them into alternative developmental pathways. The genetic demonstration that Delta-Notch signaling regulates insect neuroblast formation has been performed using *Drosophila melanogaster*: embryos lacking Delta or Notch function develop excess neural cells at the expense of epidermis [12].

In the nervous system of vertebrate embryos, *Notch* gene expression generally is associated with proliferative zones of undifferentiated cells [13,14,15,16,17,18, 19,20,21]. Scattered cells within proliferative zones express *Delta* [18, 20, 22,23,24], although, in chick, cells in S-phase of the cell cycle do not express *Delta1* suggesting that expression is limited to newly post-mitotic cells [18, 20, 23]. In frog and zebrafish neural plate, *Delta* gene expression appears to precede expression of early neuronal markers [25,26,27,28]. However, whether this indicates that proliferative cells of anamniote nervous systems express *Delta* genes is still an open question.

Newly-specified neurons may use Delta to signal to unspecified neighboring precursor cells via Notch. Such signaling could diversify neural cell fate by maintaining a pool of precursor cells that respond to other instructive signals or by promoting alternative developmental pathways. Gain-of-function experiments provide evidence for both ideas. For example, overexpression of a constitutively active form of Notch (Nac) in frog embryos led to an increase in the amount of neural tissue, even when cell division was blocked [29]. It was suggested that this excess neural tissue arose from specification of excess uncommitted precursor cells to a neural fate once levels of the overexpressed Notch protein fell. Frog retinal cells forced to express Nac maintained a neuroepithelial morphology rather than undergoing differentiation, which also suggests that Notch activation maintains cells in an undifferentiated state [16]. Similarly, overexpression of Delta proteins, which presumably activate endogenous Notch signaling, prevented retinal cell differentiation in frog and chick [20, 24] and formation of the earliest-born neurons in frog and zebrafish [25,26,27,28]. Each of these observations is consistent with the notion that Notch activity maintains cells in a proliferative, unspecified state.

More recently, several reports have raised the possibility that Notch activity promotes specification of certain cell types, particularly glial cells. Forced expression of Nac promoted formation of cells expressing markers of Müller glia in rat and zebrafish retinae [30, 31]. As Müller glia are among the last retinal cell types born, these results are consistent with the idea that Notch activity delays differentiation. However, Notch activity did not promote expression of markers of other late-born cell types in rat [30] and appeared to inhibit, rather than promote, cell proliferation in zebrafish [31]. Additionally, Nac expression in the mouse forebrain promoted formation of radial glia, the earliest specificied cell type in forebrain [32] and either permitted [33] or promoted gliogenesis in the peripheral nervous system [34].

By contrast, disruption of Notch signaling results in formation of excess early-born neurons. Antisense Notch RNA treatment or expression of a dominant negative form of Notch in chick resulted in excess ganglion cells, one of the first retinal cell types to differentiate [15]. Similarly, expression of dominant negative Delta proteins caused formation of excess early-born neurons in the retina of frog [24] and chick [20] and in the neural plate of frog [25] and zebrafish [26,27,28], concomitant with decreased cell proliferation [20] and reduced numbers of late-born neurons [28]. Mouse embryos homozygous for a targeted mutation of *Notch1* or *RBP-Jκ,* which encodes a downstream component of the Notch signaling pathway, had elevated levels of expression of neuronal markers, indicating formation of excess neurons [35]. Excess neurons also developed in mouse embryos homozygous for targeted mutation of *HES1*, a presumptive downstream effector of Notch signaling [36]. Recently, a mutant allele of the zebrafish *deltaA* (*dlA*) gene was identified and shown to cause formation of excess Rohon-Beard sensory neurons and decreased trunk neural crest progenitor cells [37, 38] and excess hair cells and decreased support cells in the ear [39].

To investigate further the role of Notch-mediated cell-cell signaling in vertebrate neural development we have tested neural pattern formation in zebrafish embryos. We show by ablating single, identified primary motor neurons that these cells normally inhibit nearby cells

from adopting the primary motor neuron fate. Our experiments lead us to conclude that in the absence of lateral inhibition the time during which neighboring cells can replace a missing cell is transient. This suggests that the temporal and spatial cues that specify neural cell fate are tightly regulated. We also show that in *dlA* mutant embryos neural precursor cells prematurely cease dividing and differentiate as early-born neurons with identities appropriate for their positions within the spinal cord and that mutant embryos have reduced numbers of late-born neurons and glia. These observations support the idea that Delta-Notch mediated lateral inhibition coordinates transition of proliferative neural precursors into specified neurons and glia of the spinal cord.

Results

Delta-Notch pathway genes are expressed in neural precursor cells

As a step toward a fuller understanding of the role that Delta-Notch signaling plays in patterning the zebrafish neural tube, we compared expression of several *delta* and *notch* genes to the distribution of neural precursor cells and differentiating neurons. We use the term, neural precursor cell, to indicate proliferative neuroepithelial cells that did not express Hu proteins [40]; we identified proliferative cells by their incorporation of BrdU. Conversely, we categorized those cells that expressed Hu proteins as post-mitotic, differentiating neurons. We examined the RNA expression patterns of *delta* and *notch* genes using a sensitive fluorescent detection method, choosing as our timepoint 24 h (hours of development at 28.5°C; [41]). At this stage, primary neurons have differentiated and some secondary neurons are postmitotic and have differentiated while others remain in the cell cycle [42]. The assays described here do not discriminate between primary and secondary neurons.

We show here expression of three *notch* genes: *notch1a* (*n1a*), *notch1b* (*n1b*) and *notch5* (*n5*) [43, 44]. Cells throughout the dorsoventral extent of the trunk neural tube expressed each of these genes. Both Hu-negative and Hu-positive cells expressed *n1a* (Figure 1A,B,C) whereas *n1b* and *n5* expression appeared to be complementary to Hu localization (Figure 1D,E,F,G,H,I), indicating that neural precursors expressed *n1a* and *n1b* but differentiating neurons did not. Particularly intense staining for each gene was evident apically, suggesting that transcripts were concentrated where the neural tube lumen will form.

We showed previously that post-mitotic primary neurons express *dlA* transiently at the 4 somite stage [28]. Many neural plate cells in excess of known post-mitotic neurons expressed *dlA* and *dlD*, suggesting that these cells include neural precursors [26,27,28]. By contrast, *dlB* expression appeared to be limited mostly to post-mitotic neurons [27]. At 24 h, cells throughout the trunk neural tube, including Hu-positive cells, expressed *dlA* (Figure 1J,K,L). Both Hu-positive and Hu-negative cells also expressed *dlB* (Figure 1M,N,O), however, apparently fewer of each class expressed *dlB* at high level than *dlA*. Some Hu-positive and many Hu-negative cells expressed *dlD* (Figure 1P,Q,R). Together, these observations suggest that a subset of neural precursors and cells specified for neuronal development express *dlA*, *dlB* and *dlD*.

To examine directly whether proliferative cells of the trunk neural tube express *delta* and *notch* genes, we exposed embryos to a brief pulse of BrdU at 24 h. BrdU-positive cells expressed each of the *notch* (data not shown) and *delta* genes we tested (Figure 2A,B,C). However, only a fraction of BrdU-positive cells expressed *delta* genes:11.2% expressed *dlB* (169 cells counted), 19.2% expressed *dlA* (228 cells counted) and 20.1% expressed *dlD* (186 cells counted). Together with the above data, we conclude that at least a portion of proliferative, neural precursor cells express each of these genes and that post-mitotic neurons express *n1a*, *dlA*, *dlB* and *dlD*. We speculate that the subset of BrdU-positive cells that express *delta* genes are those fated to exit the cell cycle following the next mitosis and we interpret our failure to detect *n1b*, *n5, dlB* and *dlD* RNA in all or some presumptive neurons as evidence that transcription of these genes is not maintained after cell cycle exit.

Lateral inhibition regulates primary motor neuron number

Widespread neural expression of *delta* and *notch* genes is consistent with the possiblity that cell-cell signaling contributes to pattern formation within the zebrafish nervous system. To examine this directly, we performed experiments designed to test whether lateral inhibition patterns primary motor neurons. Zebrafish primary motor neurons are distributed with a striking periodicity [45, 46]. In previous studies we found that *islet1* (*isl1*) expression, which marks primary motor neurons, was modified over time. Initially, contiguous cells in a row close to floorplate expressed *isl1*, whereas later, *isl1*-expressing cells were interspersed with cells that did not express *isl1* [46]. Currently, we cannot distinguish between the possibilities that alternating cells downregulate *isl1* expression or that cells that do not express *isl1* move between those that do. Nevertheless, to maintain this periodicity, *isl1*-negative cells must not transcribe *isl1*, despite being equidistant with *isl1*-positive cells from notochord and floorplate, presumed sources of signals that induce motor neuron development [47]. To test whether newly born primary motor neurons inhibit neighboring cells from adopting similar fates, we ablated identified CaP and VaP motor neurons [48] and asked

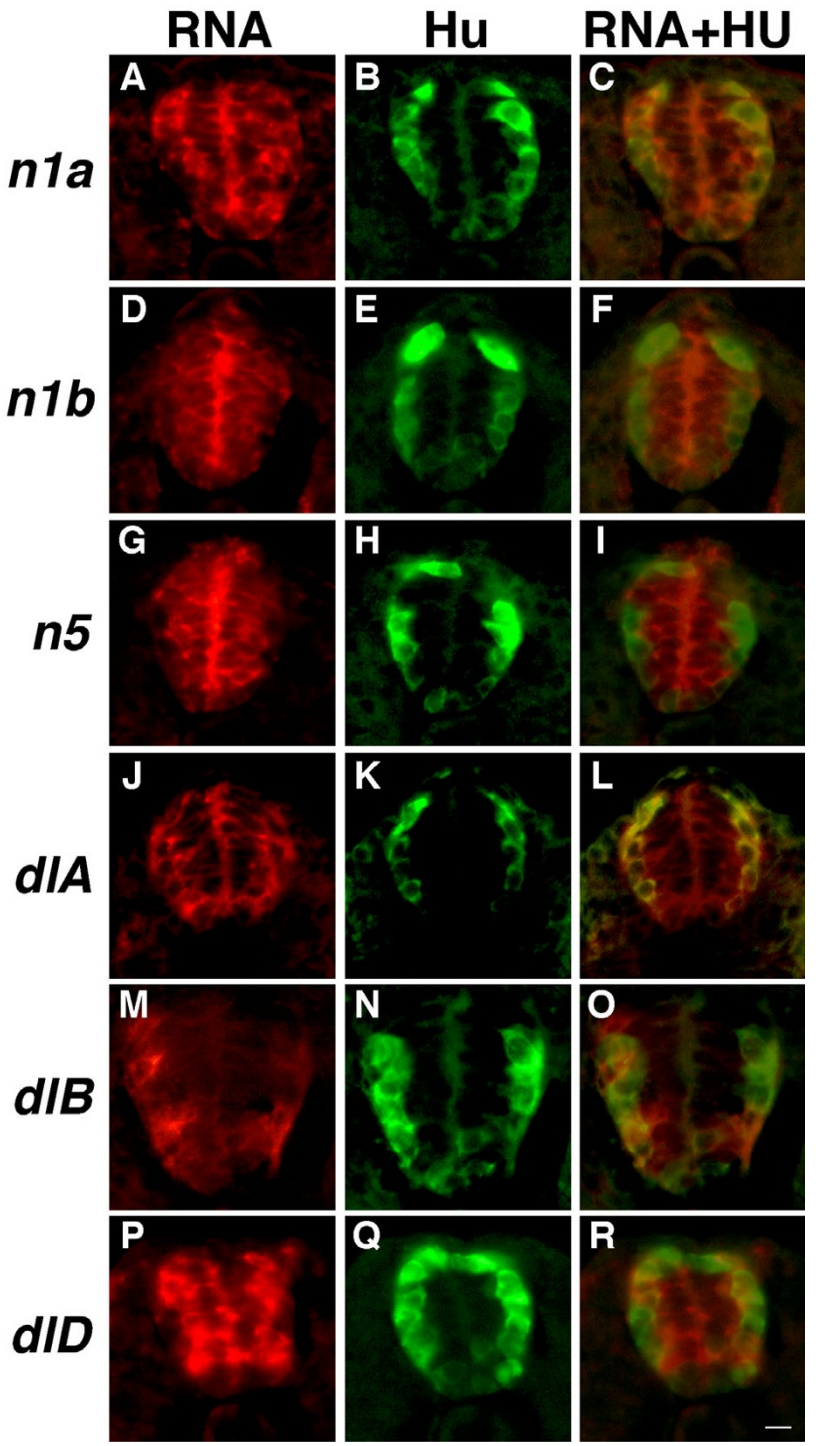

Figure 1
***notch* and *delta* gene expression in zebrafish trunk neural tube.** All images are of transverse sections through trunk neural tube. Images in each row are of the same section. First column: fluorescent detection of RNA hybridization using *notch* and *delta* probes. Second column: localization of post-mitotic neurons using anti-Hu antibody. Third column: composite images of RNA and Hu localization. Both Hu-positive and negative cells expressed *n1a* (A-C), *dlA* (J-L) and *dlB* (M-O) and *dlD* (Q-R) whereas predominantly Hu-negative cells appear to have expressed *n1b* (D-F) and *n5* (G-I). Scale bar, 10 μm.

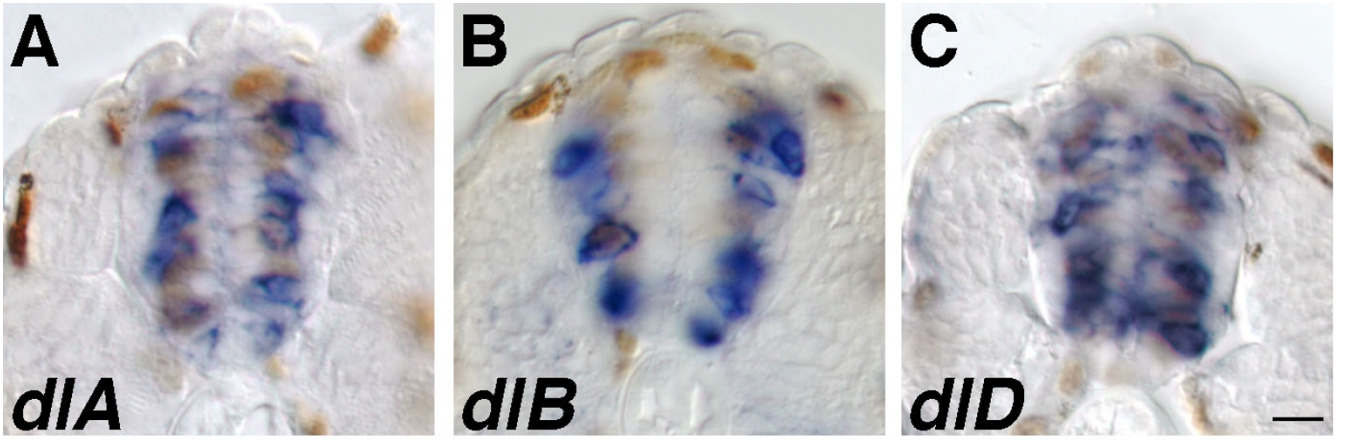

Figure 2
Proliferative neural cells express *delta* genes. Labeling to detect incorporation of BrdU during S phase of the cell cycle (brown staining) and RNA expression (blue staining). Images are of transverse sections through trunk neural tube. BrdU-labeled cells expressed *dlA* (A), *dlD* (B) and *dlB* (C). Scale bar, 10 μm.

whether these cells were replaced. Normally, CaP develops in every hemisegment, midway between adjacent somite boundaries. VaP develops in about one half the hemisegments; when present it is next to CaP. Both cells express *isl2* whereas other primary motor neurons do not [46]. Previous experiments showed that when CaP was removed from 13 somite stage embryos, it was never replaced [49]. For this experiment, we extirpated CaP and, when present, VaP from the right side of spinal hemisegment 7 (R7) in 11 somite stage embryos, 1 hour earlier in developmental time than in previous experiments [49]. We let these embryos develop until the 19 somite stage and probed for expression of *isl2*. Of a total of 13 embryos in which these ablations were performed, 11 developed a CaP or CaP and VaP in the ablated position, indicating that the ablated cells were replaced. The remaining two embryos showed no replacement. Although we do not know the normal fates of the cells that replaced ablated primary motor neurons, these observations are consistent with the possibility that, during normal development, primary motor neurons inhibit neighboring cells from adopting a primary motor neuron fate.

deltaA mutant embryos have fewer neural precursor cells and excess neurons

To further test the role of Delta-Notch mediated lateral inhibition in zebrafish neurogenesis, we examined embryos homozygous for a missense mutation of *dlA* (*dlA*^{dx2}). We previously described the effect of this mutation on development of floorplate, notochord and hypochord [37] and dorsal spinal sensory neurons and neural crest [38]. To gauge the extent to which Notch signaling is disrupted in *dlA*^{dx2} mutant embryos we examined expression of *ngn1*, a proneural gene negatively regulated by Notch activity [50]. Zebrafish neural plate stage embryos express *ngn1* in three longitudinal domains of the prospective trunk neural tube ([50]; Figure 3A), which correspond to regions that express elevated levels of *dlA* and in which the earliest neurons are born [28]. Cells within the longitudinal domains of wild-type embryos do not express *ngn1* uniformly; rather, in situ RNA hybridization revealed higher levels of *ngn1* transcripts in some cells than in others ([50]; Figure 3A). By contrast, cells within the longitudinal domains of *dlA*^{dx2} mutant embryos expressed *ngn1* at uniformly high levels (Figure 3B). Notably, the mediolateral extent of each domain appeared unchanged. We conclude that the *dlA*^{dx2} allele disrupts Notch signaling within neurogenic regions of zebrafish embryos but does not influence the formation of neurogenic domains.

In addition to elevated levels of *ngn1* expression, *dlA*^{dx2} mutant embryos have excess Rohon-Beard spinal sensory neurons and reduced numbers of trunk neural crest precursors [37, 38]. To investigate the basis for these and other neural phenotypes in *dlA*^{dx2} mutant embryos we compared the balance of proliferative neural precursor cells and post-mitotic neurons in wild-type and *dlA*^{dx2} mutant embryos. When treated with a pulse of BrdU at 24 h, wild-type embryos revealed BrdU-positive cells and a distinct, non-overlapping set of basally located *huC*-positive neurons (Figure 4A). By contrast, 24 h *dlA*^{dx2} mutant embryos had few BrdU-positive cells and a large excess of *huC*-positive cells (Figure 4B). We conclude that, in *dlA*^{dx2} mutant embryos, most neural precursor cells cease dividing and develop as neurons. Consequently, the neural precursor pool is depleted in mutant embryos and replaced by post-mitotic neurons.

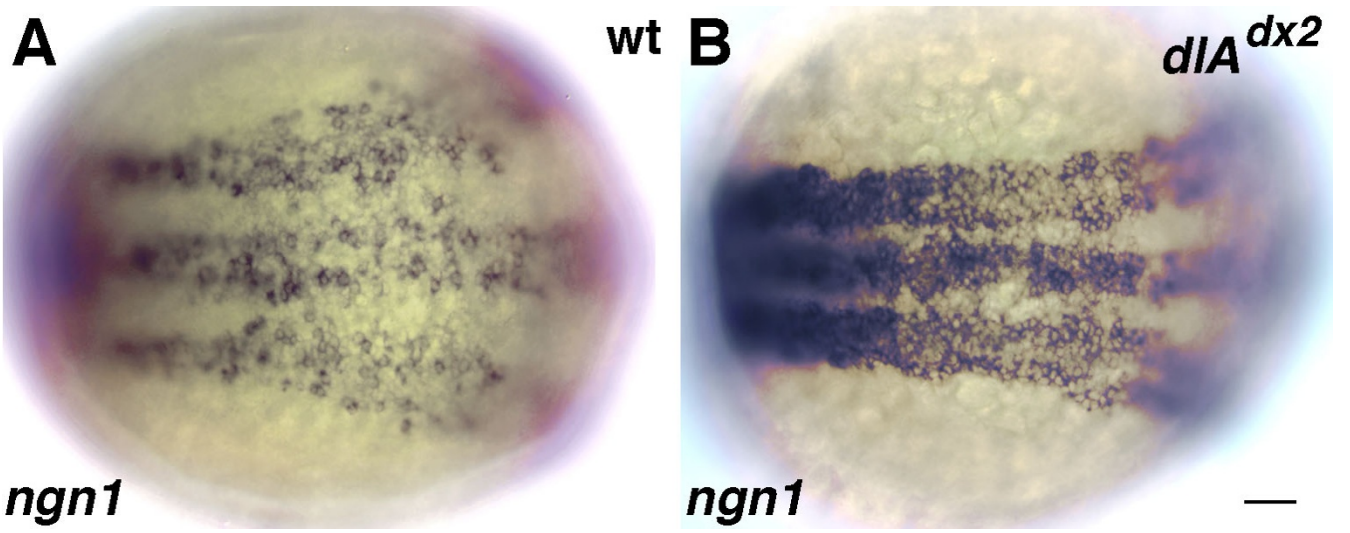

Figure 3

Notch signaling is disrupted in *dlA* ^{dx2} mutant embryos. Dorsal views, anterior to left, of neural plate stage embryos probed for expression of *ngn1*. (A) Wild-type embryo showing three longitudinal domains of *ngn1* expression. (B) *dlA*^{dx2} mutant embryo showing uniformly high level expression of *ngn1* in each longitudinal domain. This mutant embryo was slightly older than the wild-type; consequently, the lateral longitudinal *ngn1* domains are closer to the embryonic midline. Scale bar, 50 μm.

deltaA mutant embryos have excess early-specified neurons and fewer late-specified neurons and glia

The above observations indicate that neural precursor cells prematurely differentiate in *dlA*^{dx2} mutant embryos. We imagined two possible consequences for neural cell fate specification. First, all normally developing neural cell types might be specified but some might arise ahead of schedule. Second, the progenitors for cell types that normally arise late might instead develop identities appropriate for early-born cell types, resulting in an excess of some cell types and reduction of others. We previously showed that disruption of Delta-Notch signaling, by injection of RNA encoding a dominant negative form of frog Delta-1 protein into early cleavage stage zebrafish embryos, caused formation of excess early-specified primary motor neurons and reduced numbers of later-specified secondary motor neurons [28]. Our observations of *dlA*^{dx2} mutant embryos confirm and extend those results. *dlA*^{dx2} mutant embryos had excess presumptive primary motor neurons and Rohon-Beard neurons (Figure 5A,B; [37, 38]). By contrast, the number of secondary motor neurons in mutant embryos was greatly reduced (Figure 5C,D). Primary motor neurons can be distinguished by cell body position, axon projection, and gene expression [46, 51]. To learn how the *dlA*^{dx2} mutation affected specification of primary motor neuron identity, we probed embryos for expression of *isl2*, which serves as a marker for CaP and VaP, two of four identified primary motor neurons [46].18 h mutant embryos had excess *isl2*-positive cells in the ventral trunk neural tube (Figure 5E,F). These cells appeared as clusters of about 3–5 cells, located midway between adjacent somite borders, consistent with the normal position of CaP and VaP motor neurons. This indicates that disruption of Delta-Notch signaling causes development of excess primary motor neurons, which have identities appropriate for their positions in the neural tube. Previously, we showed that primary motor neuron identity is dependent upon cell body position within the ventral spinal cord [46, 52]. We conclude that Delta-Notch signaling regulates specification of appropriate numbers of primary motor neurons but does not regulate specification of motor neuronal identity.

We also investigated whether other cell types were affected in mutant embryos by examining expression of markers of various interneurons, including *lim1, lim5* and Pax2. Spinal cord cells begin to express *lim1* and *pax2* by about 14–15 h [53, 54] and *lim5* by 24 h [55] indicating that these cells are born relatively early in neurogenesis. Excess numbers of cells expressed each of these markers in *dlA*^{dx2} mutant embryos (Figure 5G,H,I,J,K,L). Notably, the dorsoventral position of cells expressing each of these markers was similar in mutant and wild-type embryos. Finally, *dlA*^{dx2} mutant embryos had greatly reduced numbers of presumptive radial glia cells, labeled by antibody zrf-1 (Figure 5M,N). Although we do not know the time or place that these cells originate in the neural tube, we do not detect expression with this marker until 30–36 h, well after the onset of primary

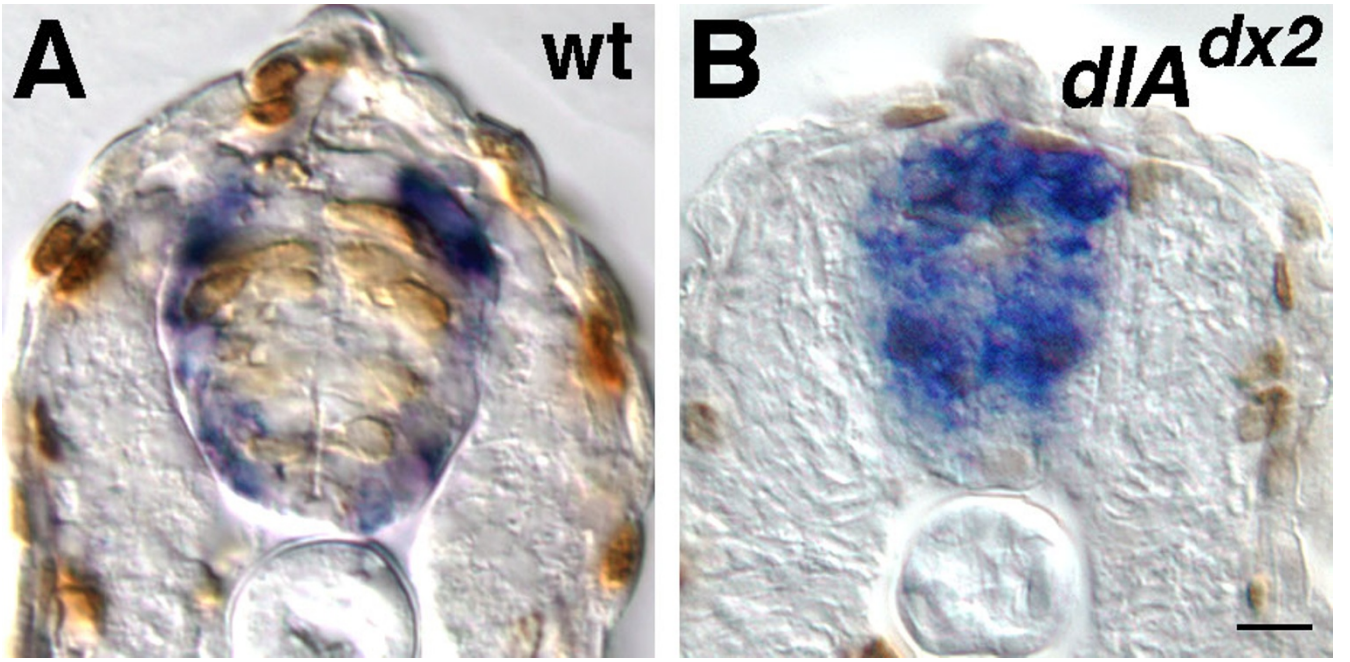

Figure 4

Neural precursor cells prematurely exit the cell cycle and develop as neurons in *dlA*^{dx2} mutant embryos. Labeling to detect proliferative cells by BrdU incorporation (brown) and post-mitotic neurons by *huC* expression (blue) on transverse sections through trunk neural tube. Both proliferative cells and post-mitotic neurons are evident in a wild-type embryo (A) whereas a mutant embryo had many more neurons and fewer proliferative cells (B). Scale bar, 10 μm.

neuron birth at about 11 h and secondary motor neuron birth at about 16 h. Together with the above BrdU results, these observations indicate that disruption of Delta-Notch signaling causes excess neural precursors to be specified for early-born neuronal fates at the expense of later-born neuronal and glial fates, without interfering with mechanisms that specify early-born neuronal identity along the dorsoventral axis.

Discussion

In zebrafish, both neural precursors and neurons express notch and delta genes

Expression of activated Notch proteins can prevent neural cell differentiation [15, 16, 20, 24, 25, 29]. In vertebrate embryonic nervous systems, proliferative undifferentiated cells express *Notch* genes [13,14,15,16,17,18,19, 21, 24]; thus, Notch activity may maintain the neural precursor state. Notably, some differentiated neural cells, such as Müller glia and radial glia, express *Notch* genes [30, 32] raising the possibility that Notch signaling does more than simply block differentiation. By contrast, only post-mitotic neural cells have been shown to express *Delta* genes [18, 20, 23]. This pattern of expression is consistent with the idea that specified, post-mitotic cells use Delta signaling to prevent neighboring cells from differentiating. However, it differs from the fly embryonic central nervous system wherein all cells of the prospective neuroectoderm express Delta with the exception of delaminated neuroblasts [56]. This indicates that, in flies, precursors express Delta and that those specified for neuroblast development downregulate its expression.

Here, we show that some zebrafish neural cells in S phase of the cell cycle and post-mitotic differentiating neurons express *delta* genes. We interpret these data to mean that a subset of proliferative neural precursors upregulate *delta* gene expression. Upregulation might engage a feedback loop mechanism, in which Notch activation in neighboring cells maintains them in a proliferative, unspecified state whereas those cells that upregulate Delta expression, and have decreased Notch activity, are driven to exit the cell cycle and to differentiate.

Zebrafish primary motor neurons may inhibit primary motor neuron fate in neighboring cells

Lateral inhibition was originally defined by functional tests in which ablation of a cell of primary fate allowed an adjacent cell to take that fate, but ablation of a cell of secondary fate had no effect [8]. Ablation experiments performed on grasshoppers showed that neuroblasts laterally inhibit neighboring cells from also developing as neuroblasts [5]. Genetic analyses of fly development then led to the view that lateral inhibition is mediated by

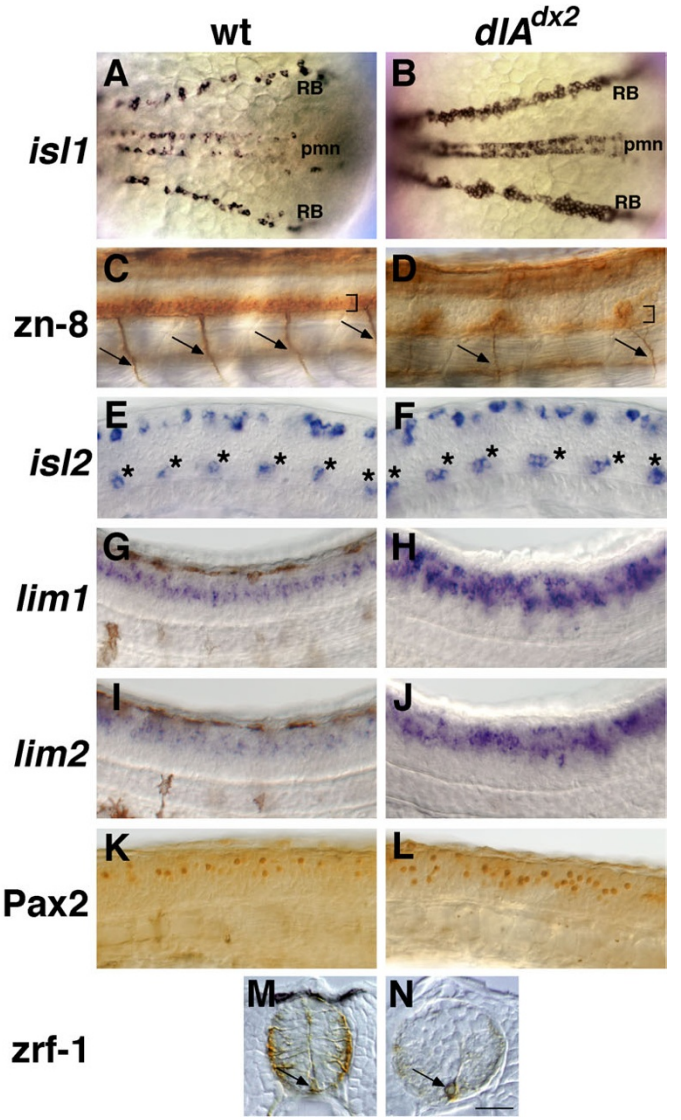

Figure 5

***dlA*^{dx2} mutant embryos develop excess early born neurons and fewer late-born cell types.** (A, B) Dorsal views, anterior to left, of neural plate stage embryos probed for *isl1* expression to reveal presumptive primary motor neurons (pmn) in medial neural plate and presumptive Rohon Beard sensory neurons (RB) in lateral neural plate. Excess primary motor neurons and Rohon Beard neurons developed in *dlA*^{dx2} mutant embryos. (C-L) Lateral views, anterior left, dorsal up. (C, D) Approximately 36 h embryos labeled with zn-8 antibody, which identifies secondary motor neuron cell bodies (bracket) and ventral nerve roots (arrows). The mutant embryo had fewer cell bodies and only two ventral nerve roots, which appear to be smaller than normal, were evident in this region of the embryo. (E, F)18 somite stage embryos probed to detect *isl2* expression, which marks CaP and VaP motor neurons in ventral spinal cord (asterisks) and Rohon Beard neurons in dorsal neural tube. One or two cells per hemisegment expressed *isl2* in ventral spinal cord of a wild-type embryo (E). Clusters of 3–5 cells per hemisegment expressed *isl2* in ventral spinal cord of the mutant embryo (F). (G, H) Approximately 30 h embryos probed for *lim1* RNA expression. The mutant embryo had many more cells that expressed *lim1* relative to wild type. Note, also, the lack of neural-crest derived melanophores, which appear brown in wild-type, in the mutant embryos shown in H and J. (I, J) Approximately 30 h embryos probed for *lim2* expression. More cells expressed *lim2* in the mutant than in wild type. (K, L) Approximately 26 h embryos labeled with antibody specific to Pax2. The mutant embryo had more Pax2-expressing cells than the wild-type. (M, N) Transverse sections of approximately 36 h embryos labeled with zrf-1 antibody, which identifies radial glial fibers. The mutant embryo completely lacked zrf-1 labeling, except for occasional cells in the ventral neural tube, which may be remnants of floorplate (arrows). Note, again, the absence of black melanophores in the mutant embryo. Scale bar, A, B 60 μm, E-L 30 μm, M, N 40 μm.

interaction of Delta and Notch proteins expressed on neighboring cells [12]. Our ablation studies, coupled with our studies of Delta and Notch expression and with analysis of a *dlA* mutation, provide evidence consistent with the possibility that similar mechanisms operate during specification of individual neural fates in a vertebrate nervous system.

Mutant and gene overexpression phenotypes in various vertebrate model systems support the notion that Delta-Notch signaling mediates lateral inhibition in the central nervous system [16, 20, 24,25,26,27,28,29, 35]. However, to our knowledge, until now direct evidence for lateral inhibition in the vertebrate central nervous system has not been reported. Previously, when primary motor neurons were ablated in 13 somite stage embryos, approximately 6 hours after birth, they were not replaced [49]. Here, we ablated primary motor neurons 1 hour earlier in developmental time and found that, in nearly every case, they were replaced. Together, our ablation results reveal an important feature of vertebrate neuronal specification: that the mechanisms that specify fate must be exquisitely timed. We view the failure to replace primary motor neurons ablated at 13 somite stage as indicating that the time at which a cell can be specified for that fate has passed, either because neighboring precursors have been specified for other fates or because the signals that specify primary motor neurons are no longer present. Future experiments, aimed at learning the fates of cells neighboring primary motor neurons may distinguish between these possibilities.

Delta-Notch signaling regulates cell proliferation and diversifies neural cell fate

Here we took advantage of the fact that dlA^{dx2} mutant zebrafish embryos survive several days after onset of neuronal differentiation to investigate how Delta-Notch signaling influences neural cell fate. We show that mutant embryos have a significant reduction in proliferative neural cells and a concomitant increase in neurons. These neurons have fates characteristic of the earliest-born neurons in zebrafish. Strikingly, the molecular identities of the excess, early-born neurons in dlA^{dx2} mutant embryos are appropriate for their positions in the trunk neural tube. This is evident across the dorsoventral axis of the neural tube as well as within localized areas of the ventral neural tube occupied by distinct motor neurons. In other words, early-born neurons are specified appropriately in dlA^{dx2} mutant embryos; what these embryos lack is the ability to regulate the number of cells specified for any particular fate.

We also showed that dlA^{dx2} mutant embryos have reduced numbers of later-born cell types including secondary motor neurons and radial glia. One possible explanation for this effect is that the premature exit of neural precursor cells from the cell cycle depletes the precursor pool. In this view, Delta-Notch signaling diversifies neural cell fate by guiding the orderly, and prolonged, transition from neural precursor cells to specified cells. Specification of a particular cellular identity might depend entirely upon the influence of instructive signals that change with time. For example, zebrafish ventral neural precursors might be specified differently by different lengths of exposure to Hedgehog (Hh) signaling. Those competent to respond to brief exposure to Hh might be specified as primary motor neurons whereas those subject to prolonged exposure prior to differentiation might develop as secondary motor neurons [57]. Notch activity might be important for specification of secondary motor neurons by holding them in an unspecified state for long enough to receive sufficient exposure to Hedgehog signaling. Disruption of Notch signaling would then allow an excess of precursor cells to respond promptly to Hedgehog signaling, producing an excess of primary motor neurons and a concomitant deficit of secondary motor neurons, precisely the phenotype of dlA^{dx2} mutant embryos we show here.

A second possibility, supported by recent publications, is that Notch activity specifies certain cell fates, in particular, glial fates. Notch activity promotes gliogenesis in forebrain, retina, and cultured neural crest [30,31,32, 34]. Our observation that dlA^{dx2} mutant embryos lack radial glia of the trunk neural tube also is consistent with this possibility. In fact, Notch activity might both hold neural precursor cells in an unspecified state and subsequently promote a subset of them to develop as glia. Conditional misexpression of activated Notch in individual precursor cells of the trunk neural tube might provide a way to distinguish between these possibilities.

Conclusions

In zebrafish, at least three *notch* genes are expressed by proliferative neural precursors and at least three *delta* genes are expressed by subsets of proliferative precursors and post-mitotic neurons. Thus, precursors fated to exit the cell cycle and differentiate may express elevated levels of *delta* genes, causing them to signal to neighboring *notch*-expressing precursors. Signaling between neighboring cells appears to regulate primary motor neuron specification: when we removed primary motor neurons soon after birth they were replaced, presumably by neighboring precursors. Genetic disruption of Delta-Notch signaling causes precursors to prematurely terminate cell division and differentiate as early-born neurons, with identities appropriate to their cell body positions, at the expense of later born neurons and glia. We propose that in the zebrafish central nervous system cell-cell interactions, mediated by Delta-Notch signaling,

help coordinate transition of proliferative precursors to specified neurons and glia.

Materials and methods

Embryos

Embryos from the University of Oregon and Vanderbilt University laboratory colonies were raised at 28.5°C. Staging was according to Kimmel et al. (1995). The *dlA*^{dx2} allele is incompletely penetrant and variably expressive [37,38,39]. Mutant embryos shown here are those exhibiting the most severe mutant phenotypes.

Cell Ablations

Individual CaP or CaP and VaP motor neurons were removed from the right side of segment 7 (R7) of 11 somite stage embryos by aspiration with a micropipette broken manually to a tip diameter of 8–12 μm, using methods described previously [52].

BrdU Labeling, in situ RNA Hybridization and Immunohistochemistry

Manually dechorionated embryos were labeled with BrdU by incubating them for 20 minutes on ice in a solution of 10 mM BrdU and 15% DMSO in Embryo Medium [58]. The BrdU solution was replaced with Embryo Medium and the embryos were incubated for 20 minutes at 28.5°C. The embryos were then anesthetized using 3-aminobenzoic acid ethyl ester and fixed in 4% paraformaldehyde. in situ RNA hybridization on BrDU-labeled embryos was carried out as described previously [59] except that the probes were not hydrolyzed. Following color development, the embryos were treated with 2 N NaOH for 1 hr, washed with PBSTx (phosphate buffered saline with 0.01% TritonX-100), blocked 1 hr in PBDTx (PBS, 1% DMSO, 0.01% TritonX-100 plus 2% sheep serum and 2 mg/ml BSA, then incubated 2 hr with anti-BrdU (monoclonal G3G4, Developmental Studies Hybridoma Bank). Detection was carried out using a biotinylated goat anti-mouse secondary antibody followed by streptavidin conjugated to peroxidase (Jackson ImmunoResearch Laboratories, Inc.). A brown precipitate was formed using Fast Dab (Sigma).

For fluorescent detection of RNA hybridization and antibody labeling, embryos were first treated for RNA hybridization and then sectioned using a cryostat microtome. The sections were incubated with monoclonal 16A11 anti-Hu antibody (University of Oregon Monoclonal Antibody Facility) and anti-digoxygenin-AP antibody (Roche). Anti-Hu labeling was detected using goat anti-mouse Alexa 488 antibody (Molecular Probes) and anti-digoxygenin-AP labeling was detected using HNPP Fluorescent Detection (Roche). Images were collected using a Spot camera (Diagnostic Instruments, Inc.) mounted on an Olympus AX70 microscope. Double-label images were created using Photoshop to merge individual red and green images.

Additional in situ RNA hybridizations were performed as described previously [59]. Additional primary antibodies used were zn-8 to detect Dmgrasp and zrf-1 (Developmental Studies Hybridoma Bank) and polyclonal rabbit anti Pax-2 (Berkeley Antibody Co.).

Acknowledgements

Thanks to C. Kimmel and R. Cornell for comments on the manuscript, M. Lardelli, J. Campos-Ortega, R. Toyama and I. Dawid, P. Blader and U. Strahle, and C. Haddon and J. Lewis for providing probes, J. Doll for help with fluorescent in situ RNA hybridization, R. Cornell for help with the ablation experiments, A. Swaims for sectioning, and members of the University of Oregon and Vanderbilt University zebrafish facility staffs for fish care. Antibody G3G4, developed by S.J. Kaufman, and antibodies zn-8 and zrf-1, developed by B. Trevarrow, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported by NIH grants NS23915 and HD22486 to J.S.E. and Vanderbilt University Start-Up Funds and NIH grant HD38118 to B.A. Additional support for the University of Oregon zebrafish facility was provided by NIH G20-RR11724, NSF STI-9602828, the MJ Murdock Charitable Trust and the WM Keck Foundation.

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