V2a and V2b neurons are generated by the final divisions of pair-producing progenitors in the zebrafish spinal cord

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The p2 progenitor domain in the ventral spinal cord gives rise to two interneuron subtypes: V2a and V2b. Delta-Notch-mediated cell-cell interactions between postmitotic immature neurons have been implicated in the segregation of neuron subtypes. However, lineage relationships between V2a and V2b neurons have not been reported. We address this issue using Tg[*vsx1*:GFP] zebrafish, a model system in which high GFP expression is initiated near the final stage of p2 progenitors. Cell fates were followed in progeny using time-lapse microscopy. Results indicate that the vast majority, if not all, of GFP-labeled p2 progenitors divide once to produce V2a/V2b neuron pairs, indicating that V2a and V2b neurons are generated by the asymmetric division of pair-producing progenitor cells. Together with evidence that Notch signaling is involved in the cell fate specification process, our results strongly suggest that Delta-Notch interactions between sister cells play a crucial role in the final outcome of these asymmetric divisions. This mechanism for determining cell fate is similar to asymmetric divisions that occur during *Drosophila* neurogenesis, where ganglion mother cells divide once to produce distinct neurons. However, unlike in *Drosophila*, the divisional axes of p2 progenitors in zebrafish were not fixed. We report that the terminal division of pair-producing progenitor cells in vertebrate neurogenesis can reproducibly produce two distinct neurons through a mechanism that may not depend on the orientation of the division axis.

KEY WORDS: Neural development, Asymmetric division, Spinal cord, Zebrafish, V2 neuron

INTRODUCTION

Neuronal diversity, one of the fundamental features of the nervous system, occurs as a result of several developmental mechanisms. In the developing vertebrate spinal cord, neural progenitor cells initially group together into distinct domains along dorsoventral axis in response to signals emanating from local organizing centers. In the ventral spinal cord, five distinct domains, p3, pMN, p2, p1 and p0, are known to be formed (Goulding and Lamar, 2000; Jessell, 2000). The next level of complexity arises within each specific domain. One mechanism that underlies this second level of neuronal diversity involves specific cell-cell interactions via Delta-Notch signaling. For example, the p2 progenitor domain generates two distinct neuronal subtypes, V2a and V2b (Karunaratne et al., 2002), and recent studies have shown that Delta-Notch signaling plays a crucial role in this segregation process (Del Barrio et al., 2007; Peng et al., 2007). Analyses of gene expression patterns, together with BrdU incorporation studies, strongly suggest that this Delta-Notch signaling occurs in postmitotic young neurons.

The role of Delta-Notch signaling in postmitotic young neurons has been extensively investigated in *Drosophila*. In this organism, neuronal precursor cells divide asymmetrically to produce proliferative precursor cells and ganglion mother cells (GMC). GMCs then undergo a terminal asymmetric division to produce two distinct neurons (Roegiers and Jan, 2004). Delta-Notch-mediated cell-cell interactions between these two sibling neurons have been shown to be crucial for proper asymmetric division (Buescher et al., 1998). A similar mechanism may facilitate the generation of V2a and V2b neurons, with these two distinct types

of neurons specified via Delta-Notch signaling between two sibling neurons arising from a single precursor. However, it is also possible that Delta-Notch signaling occurs between populations of young neurons that come from different precursors.

Distinguishing between these two potential mechanisms requires lineage analysis from the p2 progenitor stage through to final neuronal differentiation, which is difficult to accomplish in amniotes. Indeed, it is not known whether V2 neurons in amniotes are generated from the division of pair-producing progenitor cells or from cell divisions that produce progenitors and neurons. The paucity of available neuronal lineage information is a current limitation in the study of CNS development in amniotes. In our present study, we have addressed this issue using zebrafish as a model system.

MATERIALS AND METHODS

Transgenic zebrafish
Tg[*vsx1*:GFP] was constructed from zC67N1 BAC (Kimura et al., 2006; Lee et al., 2001). Tg[*huC*:Gal4-VP16] was constructed using the *huC* promoter (Higashijima et al., 2003) and Gal4-VP16 (Koster and Fraser, 2001). Tg[*UAS*:myc-notch intra] (Scheer et al., 2001) was obtained from the Zebrafish International Resource Center.

Antibodies
Primary antibodies used were guinea pig anti-Chx10, rat anti-Vsx1 (these were generated in our laboratory using bacterially-expressed proteins), rabbit anti-Scl (Qian et al., 2007), rabbit anti-GFP (Invitrogen) and mouse anti-PH3 (Upstate).

Time-lapse observations
Tg[*vsx1*:GFP] embryos at 16 hpf (hours post fertilization) were mounted in 2% low-melting-point agarose. Spinal segments 2-10 were observed every 10-30 minutes under a confocal microscope (Zeiss LSM510) at room temperature. A solution of 0.003% 3-amino benzoic acid ethyl ester was periodically added to the specimens to stop spontaneous movement. After 4 to 6 hours of observation, embryos were fixed and processed for immunohistochemistry. For figure preparation, several optical sections were stacked from which montages were generated.

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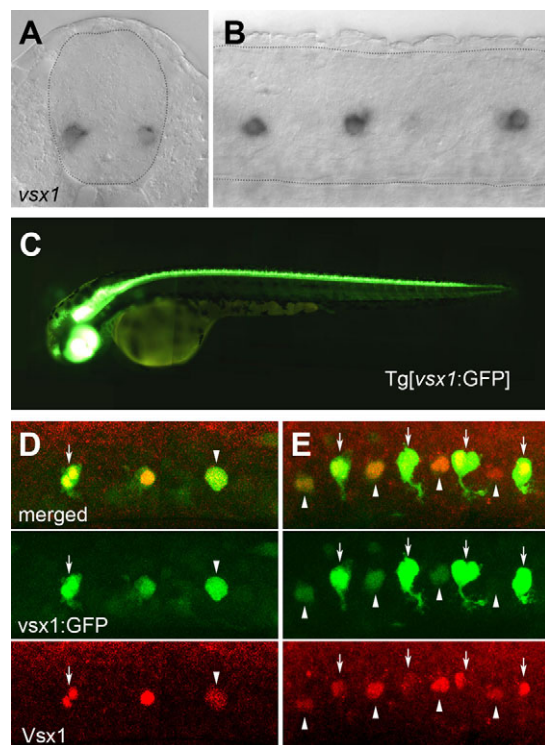*Author for correspondence (e-mail: shigashi@nips.ac.jp)

Fig. 1. *vsx1* mRNA and *vsx1*:GFP expression in spinal cord. (**A**,**B**) *vsx1* mRNA expression in 22 hpf embryos. Broken lines outline the boundary of the spinal cord. (A) Cross-section. (B) Lateral view of a whole mount. (**C**) Tg[*vsx1*:GFP] fish at 2.5 dpf. (**D**,**E**) Expression of GFP and Vsx1 in Tg[*vsx1*:GFP] embryos at (D) 17 hpf and (E) 19 hpf. Arrows indicate pairs of labeled cells. Arrowheads indicate single labeled cells.

RESULTS AND DISCUSSION
Tg[*vsx1*:GFP] zebrafish

During the course of our studies on V2 neuron development, we observed that a homeobox gene, *vsx1* (also known as *chx10.1*), was expressed in the V2 region of the spinal cord (D'Autilia et al., 2006). As shown in Fig. 1A,B (22 hpf), *vsx1* mRNA was detected in a small number of cells located near the lateral region of the spinal cord, just dorsal to the motoneuron domain (V2 region). To visualize *vsx1*-expressing cells in live animals, we generated Tg[*vsx1*:GFP] zebrafish. As shown in Fig. 1C (2.5 dpf), GFP was expressed in the spinal cord, as well as in the brain and eye, consistent with a previous report showing *vsx1* expression in these regions (Passini et al., 1998).

We focused our analyses on GFP-expressing cells during early developmental stages. Fig. 1D shows GFP expression at 17 hpf. Immunohistochemical staining with the anti-Vsx1 showed that cells expressing GFP at high levels also expressed Vsx1, indicating that *vsx1* expression was faithfully recapitulated in Tg[*vsx1*:GFP]. We then performed our subsequent analyses on these GFP-positive, *vsx1* intensely labeled cells (vsx1-GFP cells).

As GFP is stable protein, we anticipated that *vsx1*-expressing cells and their progeny would be labeled in Tg[*vsx1*:GFP]. Vsx1-GFP cells frequently presented as side-by-side pairs (arrow in Fig. 1D). As development proceeded, more vsx1-GFP cells were present (Fig. 1E; 19 hpf), and the number of labeled, paired cells increased (arrows in Fig. 1E). Vsx1 expression was transient, so some vsx1-GFP cells had lost Vsx1 expression at this stage (see Fig. S1 in the supplementary material). GFP expression was also observed in

single cells (arrowheads in Fig. 1E). The fluorescence intensity of single vsx1-GFP cells tended to be weaker than that of paired cells, suggesting that these cells had recently initiated GFP expression.

Occasionally, single spherical vsx1-GFP cells showed weak Vsx1 immunoreactivity (arrowhead in Fig. 1D), suggesting that Vsx1 proteins in these cells had spread within the soma due to the collapse of the nuclear membrane during mitosis. Consistent with this view, phospho-histone H3 (PH3) immunoreactivity (a marker of mitotic cells) was occasionally detected in these spherical cells (see Fig. S2 in the supplementary material). These observations suggested that GFP expression was initiated in single p2 progenitor cells and that the paired cells were their progeny.

Cell fate of vsx1-GFP cells

To verify this possibility, and to further investigate the ultimate fate of the paired vsx1-GFP cells, we performed time-lapse imaging followed by immunohistochemistry for markers of differentiated V2 neurons. The markers used were Chx10/Alx/Vsx2 (V2a marker) and Scl (V2b marker) (Smith et al., 2002). Cells were analyzed in the time-lapse experiments that met the following criteria: (1) initial GFP expression occurred in singly isolated cells (earlier forming pairs were excluded); (2) daughter cells expressed at least one of the V2 markers (later forming pairs without marker expression were excluded); and (3) intermingling did not occur between the two different lineages.

Representative examples from these analyses, which involved of a total of 120 cell lineages, are shown in Fig. 2A (lateral view; see Movie 1 in the supplementary material) and Fig. 2B (dorsal view; see Movie 2 in the supplementary material). The results are summarized in Fig. 2C. Two trends became evident. First, the vast majority (118 out of 120) of individual cell lineages resulted in paired cells. Second, the vast majority of these pairs (112 out of 118) consisted of V2a/V2b pairs. In six cases, one of the cells in the pair did not have detectable levels of one of the V2 neuronal markers (two V2a/ND and four V2b/ND). The absence of V2 marker expression in one of the pairs might be attributable to the difference in the timing of protein expression. In two cases, the vsx1-GFP cells directly differentiated into Chx10-positive cells without any sign of division. Thus, there were a few examples of V2a neurons that were not produced by asymmetric division, but the observation frequency of these neurons was extremely low (two out of 120). Thus, the vast majority, if not all, of the V2 neurons were generated by pair-generating progenitors that undergo a single division to produce V2a/V2b pairs.

Although vsx1-GFP expression was initiated in the p2 progenitors, it is important to note that intensely labeled cells were located near the lateral surface of the spinal cord, where the final divisions of these cells occurred (Fig. 2B). These cells thus appeared to be qualitatively distinct from general proliferative precursor cells, which have an elongated shape in the medial-lateral dimension and maintain contact with luminal side of the spinal cord (Kimmel et al., 1995). This suggests that laterally located p2 progenitors with a high level of *vsx1* expression represent an intermediate state, such that they are committed to become pair-producing progenitors.

These putative intermediate progenitors, referred to as p2 intermediate progenitors hereafter, might be analogous to the basal progenitors in mammalian cortex development, as both cell types are located in a relatively basal (outer) region of the CNS and divide once to produce neuron pairs (Haubensak et al., 2004; Noctor et al., 2004). However, this is not fully certain as basal progenitors in the cortex are derived from divisions of apical-surface-contacting neuroepithelial cells, whereas p2 intermediate progenitors may be directly derived from neural plate cells without divisions, given that

they arise as early as 16 hpf (around the stage at which the neural rod is just formed). The elucidation of how p2 intermediate progenitors arise is an important issue for future studies.
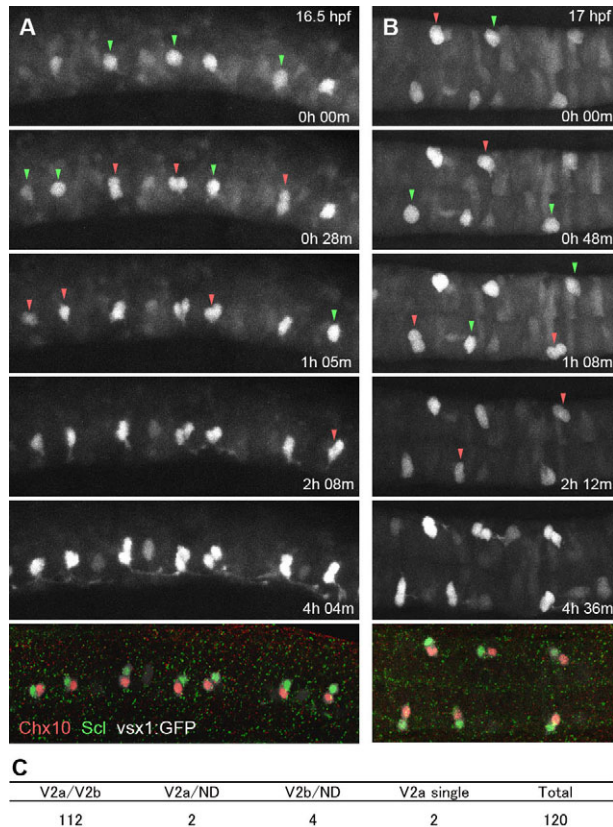

Fig. 2. Time-lapse imaging of vsx1-GFP cells showing that V2a and V2b neurons are produced as siblings. (**A**,**B**) Time-lapse imaging of vsx1-GFP cells, followed by immunohistochemical localization of Chx10 (red, V2a) and Scl (green, V2b). (A) Lateral view. (B) Dorsal view. Images are montages of stacked optical sections. Green arrowheads indicate cells prior to division. Red arrowheads indicate paired cells after division. Movies for these analyses are available in the supplementary material. (**C**) Summary of time-lapse imaging.

Mechanism of V2 differentiation

Notch signaling has recently been shown to play a crucial role in the segregation of V2a and V2b neurons (Del Barrio et al., 2007; Peng et al., 2007). These findings, along with the lineage information described above, suggest that Delta-Notch-mediated cell-cell interactions might occur between the two p2 sibling neurons and facilitate the adoption of distinct cell fates. Indeed, upon forced activation of Notch signaling, both paired vsx1-GFP cells adopted the V2b fate (see Fig. S3 in the supplementary material), consistent with the following developmental scheme: in p2 sibling neurons, one cell has relatively high-level of Notch signaling and adopts the V2b fate, whereas the other cell, containing lower activity, adopts the V2a fate.

There appears to be species-specific involvement of delta genes in V2 neuron differentiation. In amniotes, *Delta-like4* (*Dll4*) has been shown to be crucial (Del Barrio et al., 2007; Peng et al., 2007). In zebrafish, *dll4*, the ortholog of amniote *Dll4,* appeared not to be expressed in the V2 lineage (data not shown), whereas *deltaC* was expressed in p2 intermediate progenitors (see Fig. S4 in the supplementary material). Following cell division, *deltaC* was expressed at higher levels in the cell from each pair that would ultimately adopt the V2a fate (see Fig. S4 in the supplementary material). This suggests that *deltaC* is involved in cell fate specification in the zebrafish V2 lineage.

Mechanism of differential Notch signaling in V2 neurons

The apparent mode of V2 neuron differentiation in zebrafish described thus far is strikingly similar to *Drosophila* neurogenesis, in which GMCs divide once to produce two distinct neurons. Delta-Notch signaling between postmitotic sibling pairs plays a crucial role in both systems. In *Drosophila*, the division axes of GMCs are fixed, and this polarized division is presumed to be important for proper uneven distribution of Numb during GMC divisions (Buescher et al., 1998). Notch signaling is inhibited in the progeny inheriting Numb (Roegiers and Jan, 2004), thereby biasing Notch signaling levels between the two sibling cells. To determine whether a similar mechanism governs the division of p2 intermediate progenitors, divisional orientation was examined by capturing images immediately after division. The results showed that division axes were not fixed (Fig. 3A1-A4, lateral views; Fig. 3B1-B4, dorsal
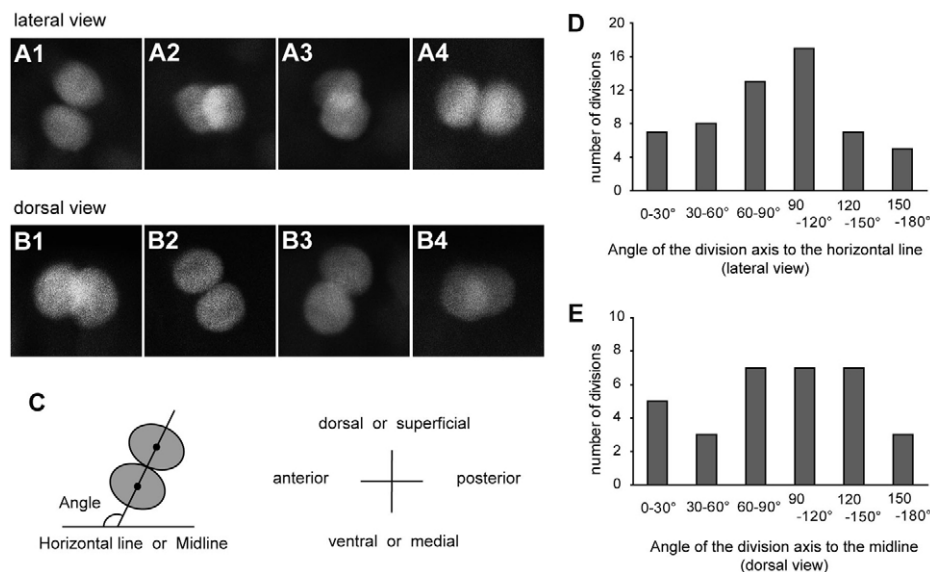

Fig. 3. Division axes of p2 intermediate progenitors are not fixed. (**A**,**B**) Confocal stacked images of vsx1-GFP sibling cells immediately after division. Embryos were analyzed at around 16-17 hpf. (A1-A4) Lateral views. Dorsal is towards the top. (B1-B4) Dorsal views. Superficial is towards the top. (**C**) Schematic of the method for division axis measurements. For each division, a line connecting the centers of the two sister cell (projection of division axis) was drawn. The angle of this line to the horizontal line (lateral views) or to the midline (dorsal views) was measured. (**D**,**E**) Quantification of the division angle.
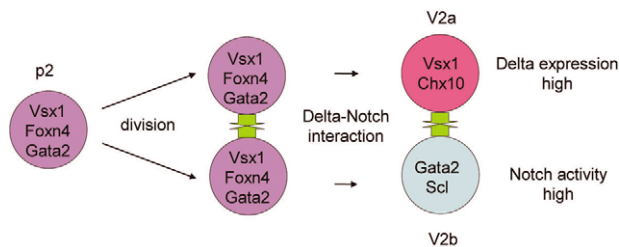
Fig. 4. Model of V2 neuron development from a p2 intermediate progenitor. In this model of one unit of lineage, a single p2 intermediate progenitor produces two sibling cells. These cells then undergo Delta-Notch-mediated cell-cell interactions, resulting in different levels of Notch activity between them. The cell with higher Notch activity develops into a V2b neuron and the sister cell with lower activity (but higher levels of Delta expression) develops into a V2a neuron.

views; embryos at around 16-17 hpf). Quantitative analyses are shown in Fig. 3D,E. Although the orientation of the mitotic axis was not completely random, the divisions occurred with various orientations. We conclude from this that the regulation of division in p2 intermediate progenitors differs from *Drosophila* GMC divisions in that the division axes are not fixed.

Additional analyses of the distribution pattern of a Numb-mCherry fusion protein in p2 intermediate progenitors showed that Numb-mCherry appeared evenly distributed during division (data not shown). The quasi-random orientations of division axes and even distribution of Numb-mCherry might suggest that there is no cell fate bias at the time of division. Asymmetry could be initiated de novo by a stochastic fluctuation in Notch signaling following division, with Delta-Notch-mediated interaction between sibling cells promoting the initial weak asymmetry. Alternatively, biasing factors may exist and the mechanism for their uneven distribution may differ from that employed in *Drosophila*. A candidate biasing factor might be *deltaC* mRNA, which is present in p2 intermediate progenitors (see Fig. S4 in the supplementary material). These transcripts (or DeltaC protein) might be unevenly distributed to the two sibling cells, either actively or stochastically.

Apart from possible involvement of asymmetry, the presence of *deltaC* in p2 intermediate progenitors suggests that genetic programs for future Delta-Notch signaling in p2 progeny have been, to some extent, predetermined in p2 intermediate progenitors. In mammals, *foxn4* has been shown to be important for the initiation of Delta-Notch signaling via the activation of *Dll4* expression, directly or indirectly (Del Barrio et al., 2007). *foxn4*, which is also expressed in p2 intermediate progenitors (see Fig. S5A in the supplementary material), may be involved in activation of *deltaC* expression in zebrafish.

In addition to the cell-cell interaction between sibling pairs, externally derived signals, such as those emanating from the floorplate, could influence cell fates. If so, there might be some correlation between cell fate and relative position of the cell in a pair. However, V2a/V2b pairs show evidence of various spatial configurations (see Fig. S6 in the supplementary material), suggesting that cell fates are not strongly influenced by extrinsic factors.

A model of V2 neuron differentiation is represented in Fig. 4 (the expression patterns of *vsx1*, *gata2* and *foxn4*, presented in Figs S1 and S5 in the supplementary material, have been incorporated). Although similar to previous models (Del Barrio et al., 2007; Peng

et al., 2007), our current model has the crucial distinguishing feature that it represents one unit of lineage. An important issue that arises from this is whether the same lineage relationship is present in mammalian V2 neurons. Based upon gene expression pattern similarities, it is possible that mammalian V2 neurons might also originate from the division of pair-producing progenitors. However, in subsequent Delta-Notch interactions, differences could arise owing to the higher cell density in the mammalian spinal cord, including possible Delta-Notch interaction between cells which are not lineage related. Nonetheless, because sibling pairs have immediate exposure to each other following division, they are perfectly positioned to initiate Delta-Notch signaling. Hence, it is likely that binary fate determination occurs between sibling cells. Clarification of this possibility will require lineage analysis of mammalian V2 neurons.

The results of our current study are in agreement with previous reports suggesting that Delta-Notch-mediated binary fate specification between sibling postmitotic neurons could occur in other regions of the vertebrate spinal cord (Mizuguchi et al., 2006; Shin et al., 2007; Wildner et al., 2006). Other reports suggest that neuronal progenitor cells derived from the mammalian cortex can divide asymmetrically to produce two uniquely differentiating postmitotic neurons in a low-cell-density culture system (Kawaguchi et al., 2004; Shen et al., 2002). As *vsx1*-expressing p2 progenitors are, to some extent, analogous to basal progenitors in mammalian cortex, Delta-Notch-mediated binary fate specification between the sibling cells of a single basal progenitor cell may also occur there.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/18/3001/DC1

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