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Function of *strawberry notch* Family Genes in the Zebrafish Brain Development

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ABSTRACTS

We previously reported embryonic expression pattern of *strawberry notch* (*sbno*) family genes, suggesting involvement in brain development. However function of *sbno* genes in the vertebrate development has not been known yet. Utilizing zebrafish embryos, we experimentally examined function of *sbno* genes during brain development in this report. Knockdown experiments of *sbno1* and *sbno2a* disrupted brain morphology, and delayed developmental alteration of gene expression. The earliest effect of loss of function of *sbno* genes on the zebrafish embryogenesis that we found here was downregulation of *otx2* expression. Knockdown of *sbno1* specifically affects regionalization along the anterior-posterior axis of the brain. These results suggest essential roles of *sbno* genes in vertebrate brain development.

We carried out a microarray screening to identify novel mouse genes which are involved in brain morphogenesis, and identified *Sbno1*, a vertebrate *strawberry notch* (*sbno)* family gene (3). Subsequently we cloned three *sbno* genes (*sbno1*, *sbno2a* and *sbno2b*) of zebrafish (31). Expression pattern of *Sbno1* (mouse) and *sbno1* and *sbno2a* (zebrafish) suggested involvement of *sbno* genes in vertebrate brain development (3, 31), but their developmental function has not been examined yet. Because knockdown experiments of gene function can be easily carried out in zebrafish embryos by injection of morpholino antisense oligonucleotide, here we examined *sbno* function utilizing zebrafish embryos. Injection of morpholinos against *sbno1* or *sbno2a* abnormalized brain morphology, and made delay in neural gene expression. Simultaneous knockdown of *sbno1* and *sbno2a* basically caused severer abnormalities than that of single knockdown of either *sbno1* or *sbno2a*, but some phenotypes were unique to one of these. These results suggest that *sbno1* and *sbno2a* are involved in a molecular mechanism, which assures temporal regulation of gene expression during zebrafish brain development.

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MATERIALS AND METHODS

Fish embryos

Wild-type zebrafish (*Danio rerio*) embryos were obtained from natural crosses of fish with the AB/India genetic background. The embryos were incubated at 28.5°C in E3 embryo medium.

Whole mount in situ hybridization

Zebrafish specimens were fixed, hybridized and stained as described previously (14). BM purple (Roche) was used as the alkaline phosphatase substrate.

Gene knockdown by injection of morpholino antisense oligonucleotide

Fertilized eggs at 1-cell stage were injected with 1nL of 0.5mM Morpholino antisense oligonucleotides (MOs). As controls, we injected similar amounts (8-16 ng) of Standard Control MO (Gene Tools, LLC). MOs targeting the ATG region of *sbno1* and *sbno2a* mRNAs were designed and synthesized by Gene Tools. Two distinct MOs were prepared for each gene and confirmed that the two MOs against the same gene cause essentially the same results, suggesting specificity of the MOs we used in this study. The MO sequences are MO1-1 (TCCGCAGGATCAGGATGTCTCCGCT) and MO1-2 (AACACGCTGTGCTGTC GGTGTCCGT) against sbno1, and MO2-1 (GCATCAGGCTCCGACCAGAAACATG) and MO2-2 (GATAGTCCTCCGTGTCCATGACAAC) against sbno2a. Mainly MO1-2 and MO2-2 were used because of their stronger effectiveness than others.

Histology

Zebrafish larvae were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). After dehydration in acetone, specimens were embedded in a plastic resin (Technovit 8100, Heraeus Kulzer GmbH & Co.). The plastic block was serially sectioned at 10µm thickness. The sections were stained by hematoxylin and eosin.

RESULTS

Normal brain morphogenesis requires *sbno* genes

Gene knockdown (KD) experiment by morpholino antisense oligonucleotide (MO) injection was employed to examine the function of *sbno1* and *sbno2a* in the zebrafish brain development. To comfirm specificity of the abnormalities, we generated two species of MO against each gene, which are complementary to different region of cDNA sequence as described in Materials and Methods. Because *sbno1* and *sbno2a* exhibited the similar expression pattern (31), we carried out not only single KD, but also double KD experiment by coinjection of MOs against *sbno1* (MO1) and *sbno2a* (MO2). As a control experiment, similar mount of control MO (Gene Tool) was injected, which gave no difference from uninjected embryos in our experiments.

Injection of *sbno* MOs did not affect spatial and temporal pattern of cell cleavage, timing of 100% epiboly and onset of the gastrulation (Fig.3). At 50% epiboly stage, surface of the animal side of the *sbno2a*-KD (Fig. 1C) and double KD (Fig. 1D) embryos became rough. The rate of increase of number of the somite did not show any distinct difference between control and *sbno*-KD embryos. Tail of double KD embryos was significantly short at the tailbud stage (Fig. 1H). In normal embryos, the hindbrain was laterally expanded making regional difference of the brain morphology along the anterior-posterior axis (Fig. 1I), while this morphogenesis was not observed in *sbno*-KD embryos (Fig. 1J,K). The double KD embryos exhibited severe morphological defect in the brain and the ventricle was not clearly recognized at tailbud stages (Fig. 1L). In 2day post fertilization (dpf) zebrafish embryos, yolk of *sbno*-KD larvae was much bigger than that of control larvae (Fig. 1M), suggesting disruption of organs required for absorption of nourishment. The tail was curved ventrally in the double KD (Fig. 1P) and *sbno2a*-KD (Fig. 1O) larvae. The pigmentation of ventral side of the eyes of *sbno*-KD larvae was poor (Fig. 1M-P). Morphology of the eyes was highly disrupted in the double KD larvae (Fig. 1T).

Differentiation of two otoliths in the otic vesicle was observed in 2 dpf normal larvae (Fig. 1W). Although increase of number of otoliths was observed in small fraction of the single KD larvae, basically *sbno*-KD reduced the number of otoliths (Table I). Probably, mild suppression of otolith differentiation resulted in fragmentation of the otoliths by chance. Almost all double KD larvae did not have otolith (Fig. 1U). Thus, *sbno*-KD suppressed differentiation of the otoliths.

Table I. The percentages of the appearance of number of the otoliths in each inner ear of MO injected larvae were summarized in the table I.

Percent of otoliths				
Number of otoliths	0	1	2	3
Control- MO	0%	0%	100%	0%
Sno1-MO	0%	43%	46%	11%
Sno2a-MO	8%	29%	55%	8%
Sno1,2a-MO	93%	7%	0%	0%

Serial sections of MO-injected 2 dpf larvae were made for detailed observation of brain abnormalities. In single KD embryos, the ventricle was expanded and the neuroepithelium was thinner (Fig. 2B,C), compared to the normal counterpart (Fig. 2A). Characteristic morphogenesis was observed in the midbrain of normal larvae (Fig. 2Ac), whereas it was not observed in *sbno*-KD larvae (Fig. 2Bc, Cc, Dc). Cells dissociated from the neuroepithelium were observed in dorsal side of the ventricule of the *sbno2a*-KD and the double KD larvae (Fig. 2Ca, Cb). In the double KD larvae, the ventricle was narrow and the neuroepithelium was thicker than the normal counterpart (Fig. 2D). The brain size of double KD larvae was clearly smaller along both the dorsal-ventral and anterior-posterior axes than that of normal larvae (Fig. 2). The surface of the eye ball of normal larvae is occupied by single layered cells and no cell was observed inside the lens (Fig. 2Ab), but there was ectopic cells in the lens of double KD larvae (Fig. 2Db, Dc).

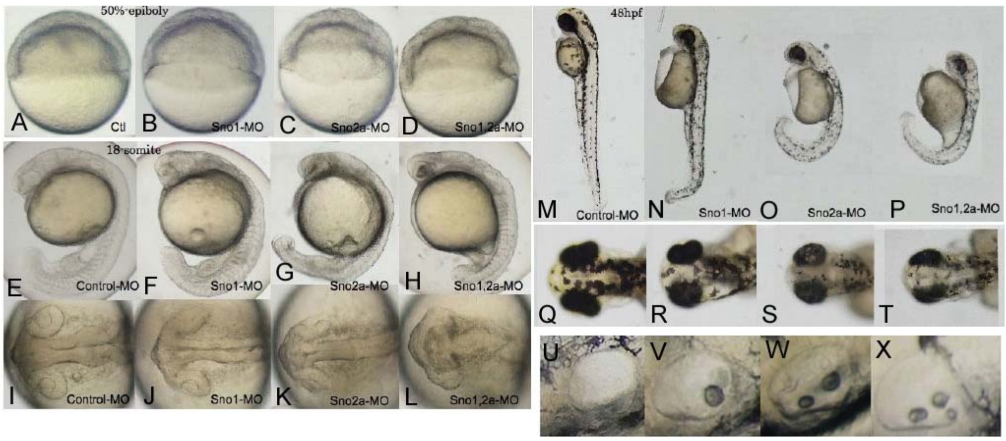

Fig. 1. Morphology of the morpholino antisense oligonucleotide-injected embryos.
Fertilized eggs were injected with non-specific control MO (A, E, I, M, Q), MO against sbno1 (MO1) (B, F, J, N, R), MO against sbno2a (MO2) (C, G, K, O, S), or MO1 and MO2 (D, H, L, P, T). The living embryos were photographed at 50% epiboly (A-D), 18-somite stage (E-L), and 48 hours post fertilization (hpf) (M-T). The lateral view of the embryos are shown, where the dorsal is to the right in A-H and M-P. The dorsal view of the embryos (I-L) and larvae (Q-T) is shown. The otoliths in the inner ear of 48 hpf larvae is shown at high magnification (U-X).

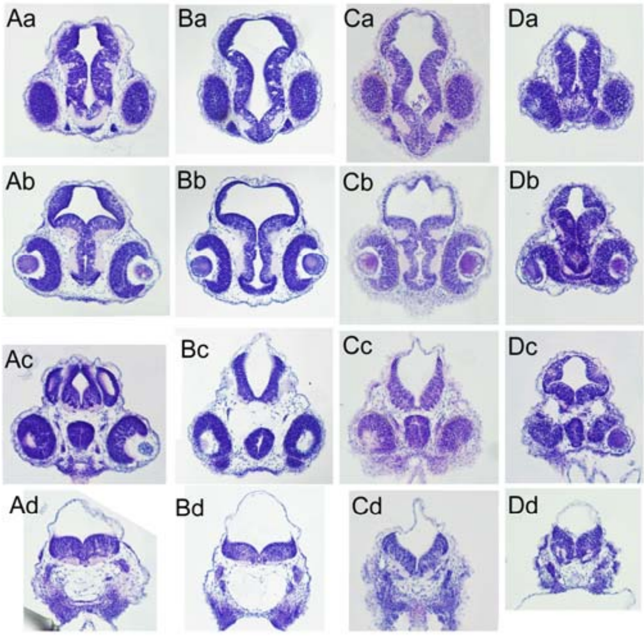

Fig. 2. Serial section of the *sbno* knockdown larvae
Larvae were injected with non-specific control MO (the series indicated by large letter "A"), MO against sbno1 (MO1) (the series indicated by large letter "B"), MO against sbno2a (MO2) (the series indicated by large letter "C"), or MO1 and MO2 (the series indicated by large letter "D"). The larvae embedded in plastic block were sectioned at 10 μ m thickness.

Effects of *sbno* knockdown on early genes expression

Because MO injection disrupted brain morphology, we examined expression of genes involved in neural development. The earliest event of neural development is the neural induction, by which dorsal part of the ectoderm takes neural fate by function of the organizer during gastrulating stage. Thus, we examined expression of genes that are essential for the organizer function at the onset of gastrulation (4 hpf) by whole mount *in situ* hybridization. However, obvious difference was not observed between control and *sbno*-KD embryos in the expression of *dharma* (34) (Fig. 3 A-D) and *chordin* (23) (Fig. 3E-H). Expression of *sox3*, one of the earliest genes expressed in the presumptive neuroectoderm (26, 27, 29, 36) was examined at dome (4 hpf) and 80% epiboly (8 hpf) stages, and significant difference in *sox3* expression was not observed between control (Fig. 3I,M) and *sbno*-KD embryos (Fig. 3N-T). *otx2* is also one of the conserved earliest genes expressed in the presumptive neuroectoderm (18), and essential for the normal brain development (1, 21, 24). Expression of *otx2* was detected in the dorsal part of the animal hemisphere of the control embryos at the dome stage (Fig. 3Q). Expression of *otx2* was slightly reduced in *sbno1*-KD embryos (Fig. 3R), very faint in *sbno2a*-KD embryos (Fig. 3S), and abolished in double KD embryos (Fig. 3T). However, there was no significant difference in *otx2* expression between control and KD embryos at 80% epiboly stage (Fig. 3U-X). These observations suggest that *sbno*-KD delay the onset of *otx2* expression. Because *dharma* is a transiently expressed gene (34), temporally normal expression of *dharma* in *sbno*-KD embryos implies that developmental process of the knockdown embryos was not generally delayed. This is consistent to the observations that the timing of early cell cleavage, the onset of epiboly and gastrulation was comparable among control and *sbno*-KD embryos (Fig. 1). Thus, it is likely that *otx2* is one of the earliest gene affected by *sbno*-KD.

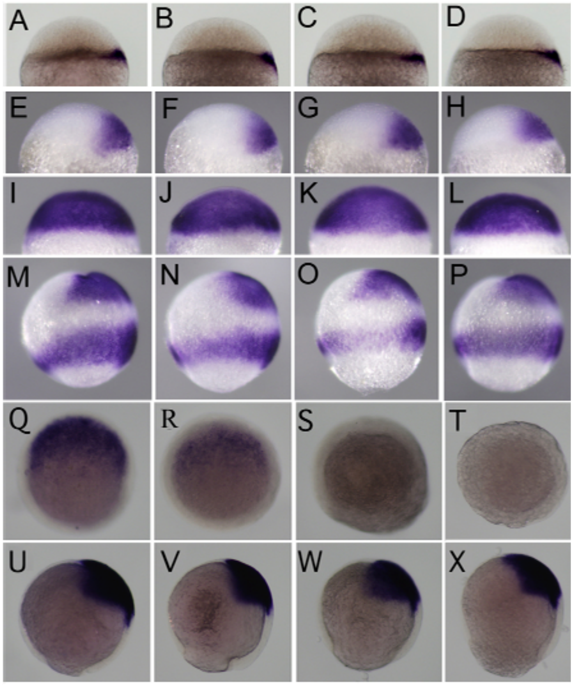

Fig. 3. Effects of *sbno* knockdown on the expression of neural inducers and early neuroectodermal markers. Expression of the Spemann organizer genes, *dharma* (A, B, C, D) and *chordin* (E, F, G, H), and early neuroectodermal markers, *sox3* (I-P) and *otx2* (Q-X) was detected by whole mount *in situ* hybridization in the control MO (A, E, I, M, Q, U), MO against *sbno1* (MO1) (B, F, J, N, R, V), MO against *sbno2a* (MO2) (C, G, K, O, S, W), or MO1 and MO2 (D, H, L, P, T, X) injected embryos at dome stage (4 hpf) (A-L, Q-T), and 80% epiboly stage (M-P, U-X). The lateral view was shown except for Q-T which are shown from animal pole of the embryos. The dorsal is to the right except of Q-T, in which dorsal is to the top.

Expression of *delta* and *HuC* genes in the brain affected by *sbno* knockdown

In the retinal development of *Drosophila*, *sbno* is involved in transcriptional regulation of *Delta* gene (32). Thus, we examined expression of four *delta* genes of zebrafish. Because *deltaA* and *deltaB* of zebrafish are early markers for neuronal differentiation (9), we also examined expression of *HuC*, the definitive neuron marker (15).

In 5-prime stage embryos, *HuC* expression marked the upper and lower rhombic lips, the prethalamus, the thalamus, the hypothalamus, and the telencephalon (Fig. 4A). While *HuC* expression in the telencephalon was not significantly affected, that in the thalamic regions became very faint in *sbno*-KD embryos (Fig. 4F, K, P). Since expression of *deltaA* in the developing brain starts earlier than that of *HuC*, *delta A* expression is more densely observed than that of *HuC* in a similar spatial pattern (Fig. 4B). Expression of *deltaA* was reduced in the thalamus and abolished in the tectum by *sbno*-KD (Fig. 4G, L, Q). Effect of *sbno*-KD on *deltaB* expression was similar to the case of *deltaA* (Fig. 4C, H, M, R). Expression *deltaC* and *deltaD* in the telencephalon of *sbno*-KD embryos was stronger than that in control embryos (Fig. 4D, I, N, S). Because expression of *deltaC* and *deltaD* in the telencephalon was gradually reduced from around 5-prime stage (n= 9; data not shown), strong expression of these genes in this brain region of *sbno*-KD embryos does not suggest enhancement of gene expression, but it likely suggests delay of developmental change of expression of these genes. Expression of *HuC* and four *delta* genes indicated that the morphogenesis of the upper rhombic lip is suppressed in the *sbno*-KD embryos at this stage.

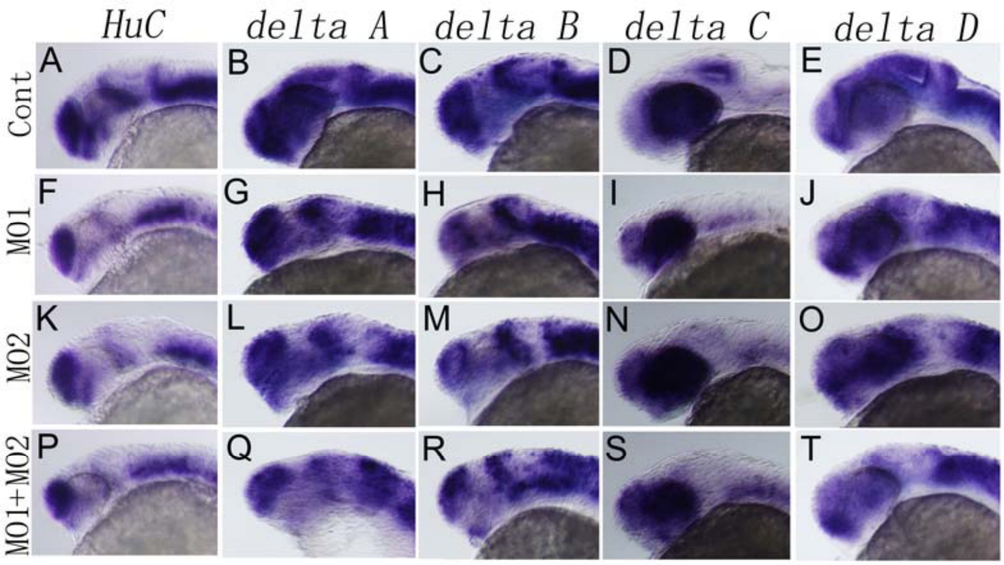

Fig. 4. Effects of *sbno* knockdown on the expression of early neuronal genes in the brain. The zebrafish embryos injected with control MO (A-E), MO1 (F-J), MO2 (K-O), or MO1 and MO2 (P-T) were fixed at 24 hpf and hybridized to the RNA probe against *HuC* (A, F, K, P), *deltaA* (B, G, L, Q), *deltaB* (C, H, M, R), *deltaC* (D, I, N, S), or *deltaD* (E, J, O, T). Lateral view of the larvae is shown. Anterior is to the left.

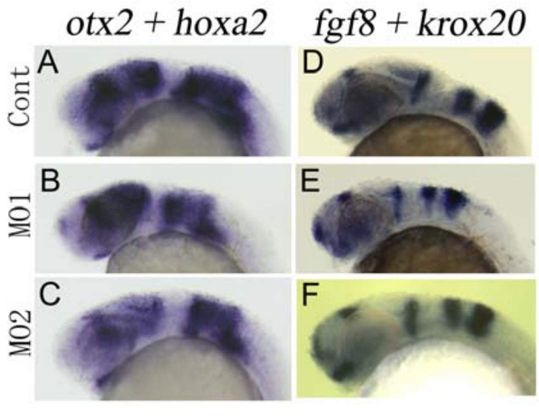

Fig. 5. Effect of *sbno* knockdown on regionalization of the neural tube along the anterior-posterior axis. Expression of *otx2* and *hoxa2* (A-C) or *fgf8* and *krox20* (D-F) was simultaneously detected in the 24 hpf embyos injected with control MO (A, D), MO1 (B, E), MO2 (C, F). Lateral view of the larvae is shown. Anterior is to the left.

Knockdown *sbno1* specifically narrowed midbrain region

Since spatial expression pattern of *sbno1* is different from that of *sbno2a* along the anterior-posterior (AP) axis (31), we investigated effects of *sbno*-KD on AP markers of the brain. In 30-somite (24 hpf) stage, *otx2* is expressed in the dorsal thalamus and the midbrain regions (30), and *hoxa2* is expressed in rhombomere 2, 3, 4, and 5 (11). There was not significant difference in spatial expression pattern of these genes between control and *sbno2a*-KD embryos (Fig. 5A, C), while *hoxa2* expressing region and posterior boundary of *otx2* were anteriorly shifted in *sbno1*-KD embryos (Fig. 5B). Anterior boundary of *otx2* was similar in control and *sbno*-KD embryos (Fig. 5A-C). Expression of *fgf8* in the isthmus and *krox20* in the rhombomere 2 and 4 was similar in control and *sbno2a*-KD embryos (Fig. 5D, F), whereas it was anteriorly shifted in *sbno1*-KD embryos (Fig. 5E). Expression of *HuC* (Fig. 2) also indicated anterior shift of gene expression in the hindbrain of *sbno1*-KD embryos (Fig. 4F). These observations suggest that *sbno1*-KD narrowed *otx2*-expressing region, which is compartible with the predominant expression of *sbno1* in *otx2* expressing region (31).

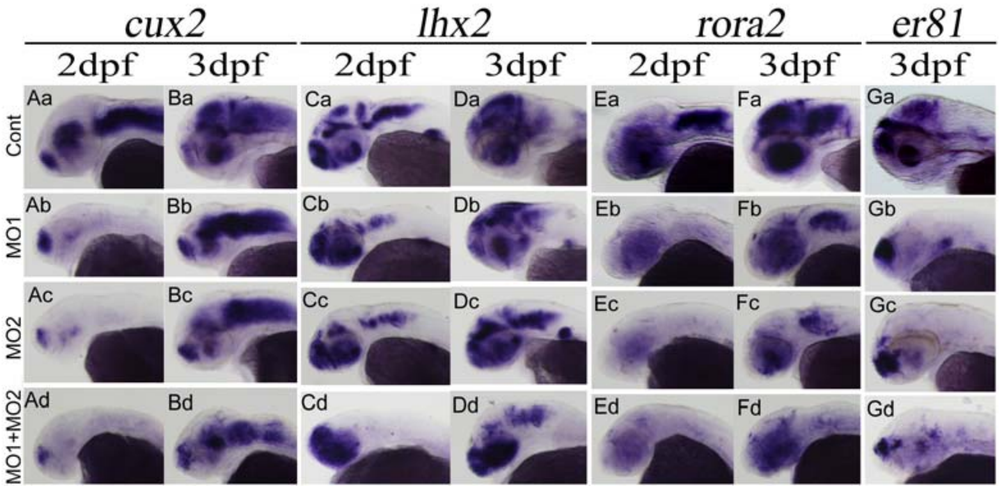

Fig. 6. Effects of *sbno* knockdown on late brain gene expression. Expression of *cux2* (A,B), *lhx2* (C,D), *rora2* (E,F) and *er81* (G,G) was examined in the larvae at 2 dpf (A,C,E) and 3 dpf (B, D, G). The larvae were injected with control MO (indicated by small letter "a"), MO1 (indicated by small letter "b"), MO2 (indicated by small letter "c"), or MO1 and MO2 (indicated by small letter "d"). Lateral view of the larvae is shown. Anterior is to the left.

Effects of *sbno*-knockdown on late neural genes

Our previous report (3) suggested possible involvement of *Sbno1* in the laminar formation in the development of the mouse cerebral cortex, Although zebrafish forebrain does not exhibit lamination, we expected that conserved function of *Sbno* family genes could be predicted by examining expression of zebrafish genes, of which homologues are essential for laminar development of the mouse cerebral cortex.

In the 2 dpf *sbno*-KD larvae, *cux2* expression in the telencephalon and eyes remained weakly, and expression in the other regions was almost abolished (Fig. 6Aa-d). Expression of *cux2* in the telencephalon and the hindbrain became weak in 3 dpf normally, whereas it was strong in the *sbno*-KD larvae (Fig. 6Ba-d). Interestingly, *cux2* expression in the 3dpf *sbno1*-KD larvae was similar to that in the 2 dpf normal larvae (Fig. 6Aa, Bb-d). Similarities were also observed between *lhx2* and *rora2* expression patterns of normal 2 dpf larvae and *sbno*-KD 3dpf larvae. For example, expression of *lhx2* and *rora2*in the posterior hindbrain became very weak in the normal 3 dpf larvae (Fig. 6Ca, Da, Ea), whereas expression of *lhx2* and *rora2* was strongly detected in the hindbrain region of *sbno*-KD 3dpf larvae (Fig. 6 Dc, Dd, Fb, Fc). In the normal 2 dpf larvae, expression of *er81* was detected in habenular nucleus and weakly in the tectum, and *sbno*-KD abolished *er81* expression completely (data not shown). In 3 dpf *sbno*-KD larvae, dense expression of *er81* was detected in the dorsal telencephalon, whereas the expression in other brain regions was very faint (Fig. 6Gb-d).

DISCUSSION

In the previously study we showed that sbno1 and *sbno2a* are expressed in the developing CNS during zebrafish embryogenesis (31). Although sbno2a and sbno2b proteins (Sbno2 homologues of zebrafish) are similar in their amino acid sequence, they exhibited completely different gene expression pattern (31). In this report, single KD of either *sbno2a* or *sbno1* abnormalized zebrafish embryogenesis, indicating that both *sbno1* and *sbno2a* genes are required quantitatively for normal embryogenesis. Specific effect of *sbno1*-KD on the presumptive midbrain region is consistent to the fact that the presumptive midbrain region strongly expresses *sbno1*. Thus, sbno1 and sbno2a have likely redundant function biochemically, but also have unique function based on the gene expression pattern.

Possible interaction of Notch signal and sbno proteins in zebrafish development

Bray (4) suggested three different functions of Notch signal, *i.e.*, lateral inhibition, lineage decisions, and boundary making (induction). In Drosophila, sbno is involved in inductive events in retinal cell differentiation (33) and in boundary formation in Drosophila wing development (25) but not in lateral inhibition (5, 18, 20). Our results in zebrafish experimental system are in good correspondence with these Drosophila studies as below.

The lateral inhibition event is essential for determination of proper number of neuronal differentiation in both vertebrates and Drosophila. Although disruption of this developmental event is one of crucial abnormalities of Notch signal mutants, the lateral inhibition is normaly observed in Drosophila *sbno* mutant (5). The zebrafish *mib* mutant suggests involvement of Notch signal in lateral inhibition which is required for normal primary neuron differentiation (12), but significant abnormality was not observed in the number of primary neurons marked by *HuC*, *deltaA*, or *islet1* expression in *sbno*-KD embryos (data not shown). We previously confirmed binding between sbno1 and Su(H) proteins of zebrafish by immunoprecipitation assay (31). Because Su(H) is required for normal lateral inhibition of zebrafish (7), it is likely that function of Su(H) in the lateral inhibition does not involve sbno.

The defects in the somitogenesis are the characteristic phenotype observed in all of the Notch pathway mutants of zebrafish (35). In sbno-KD embryos, formation of somite boundaries underwent in a normal time course, but the chevron-shaped morphology of each somite was transiently disrupted. The otolith differentiation involves Notch signal (10, 28), and *sbno*-KD larvae lost the otoliths. These observed abnormalities of *sbno*-KD suggest possible interaction of sbno and Notch function in some developmental events which involve lineage decision and boundary making.

Genetic interactions between Notch signal and chromatin remodeling mechanism have been suggesed previously. Spt6 protein has nucleosome assembly function, affecting function of SNF/SWI chromatin remodeling complex in yeast (8). Spt6 can bind to Notch receptor in nematode. Interestingly, an *spt6* mutant and *sbno* morphants of zebrafish are very similar in terms of morphology and gene expression profile (16). Overexpression of Brg gave similar phenotype to that of Delta-Notch mutants (2). Immunoprecipitation assay indicated that SWI/SNF binds to CBF-1 (a vertebrate homologue of Su(H)) (13), and Notch intracellular domain binds to Brm (6). Furthermore, Baf60c is required for Notch dependent transcriptional activation of Nodal gene in mouse and zebrafish embryos (32). Thus, it is interesting to study a possibility that sbno is involved in chromatin remodeling mechanisms.

Regulation of brain gene expression by sbno

We found that *sbno*-KD suppressed expression of *otx2*, the earliest gene to be expressed in the presumptive neural ectoderm (19, 22, 24), in the 50% epiboly stage embryos. Because *dharma*, an organizer marker is very transiently expressed at this stage (34), this is a good marker to show that *sbno*-KD is specifically affected on normal gene expression. Normal expression of the organizer markers suggests that the neural induction by the dorsal mesoderm takes place normally in *sbno*-KD embryos and a possibility that sbno is directly involved in transcriptional regulation of *otx2*.

Loss of function of *sbno* genes not only delay onset of gene expression, but also delays reduction of expression of genes. The delay of brain gene expression in the *sbno*-KD was 2 or 3 hours at gastrula stage, whereas it became more than 24 hours at 3 dpf. Taking all of these observation into account, *sbno* may be required for progression of developmental change of gene expression.

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