PlexinA4 is necessary as a downstream target of Islet2 to mediate Slit signaling for promotion of sensory axon branching

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Summary

Slit is a secreted protein known to repulse the growth cones of commissural neurons. By contrast, Slit also promotes elongation and branching of axons of sensory neurons. The reason why different neurons respond to Slit in different ways is largely unknown. Islet2 is a LIM/homeodomain-type transcription factor that specifically regulates elongation and branching of the peripheral axons of the primary sensory neurons in zebrafish embryos. We found that PlexinA4, a transmembrane protein known to be a co-receptor for class III semaphorins, acts downstream of Islet2 to promote branching of the peripheral axons of the primary sensory neurons. Intriguingly, repression of PlexinA4 function by injection of the antisense morpholino oligonucleotide specific to PlexinA4 or by overexpression of the dominant-negative variant of PlexinA4 counteracted the effects of overexpression of Slit2 to induce branching of the peripheral axons of the primary sensory neurons in zebrafish embryos, suggesting involvement of PlexinA4 in the Slit signaling cascades for promotion of axonal branching of the sensory neurons. Colocalized expression of Robo, a receptor for Slit2, and PlexinA4 is observed not only in the primary sensory neurons of zebrafish embryos but also in the dendrites of the pyramidal neurons of the cortex of the mammals, and may be important for promoting the branching of either axons or dendrites in response to Slit, as opposed to the growth cone collapse.

Key words: Zebrafish, PlexinA4, Slit, Islet2, LIM/homeodomain protein, Axon branching, Zebrafish

Introduction

During development, growth cones of axons are guided by both attractive and repulsive environmental cues, such as secreted or extracellular matrix molecules to reach the correct targets. Slit is known to be expressed along the midline of the central nervous system, and is characterized as a midline chemorepellent for commissural axons as a extracellular ligand for the Roundabout (Robo) transmembrane repulsive receptor that is expressed in the growth cones of commissural neurons in *Drosophila melanogaster* (Kidd et al., 1999). The vertebrate homologues of the *Drosophila Slit* gene, *Slit1*, *Slit2* and *Slit3*, have been cloned in mammals, zebrafish and chicken (Holmes et al., 1998; Itoh et al., 1998; Brose et al., 1999; Vargesson et al., 2001). In zebrafish, both Slit and Robo homologues have been identified (Yeo et al., 2001; Lee et al., 2001; Challa et al., 2001; Hutson et al., 2003). In vertebrates, Slit proteins also act as repulsive and collapsing factors for olfactory bulb, spinal motor, hippocampal and retinal axons (Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000). Slit proteins also repel tangentially migrating interneurons in the mouse telencephalon and mesodermal cells in zebrafish (Hu, 1999; Zhu et al., 1999; Yeo et al., 2001). Nevertheless, they have some positive effects: Slit2 is proteolytically cleaved, and N-terminal fragment of Slit2 stimulates the formation of axon collateral branches by NGF-responsive neurons of the dorsal root ganglia (DRG) (Wang et al., 1999). We have also demonstrated the pleiotropic functions of Slit in vivo by overexpression of Slit2 in transgenic zebrafish embryos. Slit2 overexpression severely affected the behavior of the Robo-positive neurons [i.e. the commissural reticulospinal neurons (Mauthner neurons)], promoted branching of the peripheral axons of the Rohon-Beard neurons and the trigeminal sensory ganglion neurons, and induced defasciculation of the medial longitudinal fascicles (Yeo et al., 2004; Lee et al., 2001). In addition, Slit2 overexpression caused defasciculation and deflection of the central axons of the trigeminal sensory ganglion neurons from the hindbrain entry point (Yeo et al., 2004) (Fig. 7F). However, the reason why different neurons respond to Slit in different ways is largely unknown.

We and others have previously reported that Islet2, a LIM/homeodomain-type transcription factor of the Islet1 family (Islet1, Islet2 and Islet3), is expressed in the primary sensory neurons and the caudal primary motoneurons (CaP) in zebrafish embryos (Inoue et al., 1994; Appel et al., 1995; Tokumoto et al., 1995). Our previous study revealed that the proteins that consist only of the LIM domains of Islet2 or Islet3 (LIM^{Isl-2} or LIM^{Isl-3}) function as a dominant-negative variant by inhibiting the heterotetrameric complex formation by Islet2 or Islet3 with homodimers of the LIM domain binding (Ldb) proteins (Kikuchi et al., 1997; Segawa et al., 2001). Overexpression of LIM^{Isl-2} causes defects in axonal outgrowth, positioning error and abnormal transmitter expression in the Islet2-expressing primary sensory neurons and CaP. Especially in the Rohon-Beard neurons and the trigeminal sensory ganglion neurons, the expression of Islet2 itself is downregulated, and their peripheral axons are completely eliminated. By contrast, the central axons extend normally in the dorsal part of the central nervous system (CNS) as in the wild-type embryos (Segawa et al., 2001). These results indicate that Islet2 is involved selectively in outgrowth and branching of the peripheral axons of the primary sensory neurons.

As Islet2 is a transcription factor, downstream target genes of Islet2 must be involved in this particular process. To determine the molecular mechanisms for the asymmetric development (peripheral versus central axons) of the primary sensory neurons and its control by Islet2 in zebrafish embryos, we focused on one of the cDNA fragments (D204) that was originally identified as a consequence of ordered differential display (ODD) screening, in order to identify the genes specifically downregulated in the tissue around the midbrain-hindbrain boundary (MHB) by overexpression of LIM^{Isl-3} in the embryo (Hirate et al., 2001). Besides expression in the midbrain, D204 is normally expressed in Rohon-Beard neurons and the trigeminal sensory ganglion neurons, and its expression in these primary sensory neurons is downregulated by overexpression of either LIM^{Isl-3} or LIM^{Isl-2} (Fig. 1A,A′,C,C′), suggesting that the gene encoded by D204 may act as a downstream target of Islet2.

We show that D204 encodes zebrafish PlexinA4. Loss of function of PlexinA4 significantly reduced branching of the peripheral axons of Rohon-Beard neurons. We further show that this phenotype is opposite to the excessive branching induced by overexpression of Slit2. In the embryos ubiquitously overexpressing Slit2, the central axons of the trigeminal sensory ganglion neurons became defasciculated and failed to enter the hindbrain. These defects were rescued by injection of antisense morpholino oligonucleotide (AMO) for *plexinA4* or overexpression of the dominant-negative form of PlexinA4 (dnPlexinA4) in the same Slit2-overexpressing embryos. Therefore, our study indicates possible interaction of PlexinA4 with Slit signaling for axonal branching in the primary sensory neurons.

Materials and methods

Zebrafish maintenance and strains

Zebrafish were maintained as described by Westerfield (Westerfield, 2000). The embryos were staged according to Kimmel et al. (Kimmel et al., 1995). The embryos of the transgenic line *Tg(α-actin:GFP)* (Higashijima et al., 1997) were found to express GFP in Mauthner neurons and other segmentally distributed reticulospinal neurons in the hindbrain (H.W. and H.O., unpublished).

Isolation RNA and cDNA library construction

Total RNA from 22-26 hours post fertilization (22-26 hpf) zebrafish embryos was isolated by using ULTRASPEC RNA isolation kit (BIOTECX Laboratories) and poly(A)$^+$RNA was selected using oligo(dT) DYNAbeads (Dynal Biotech). Double-stranded cDNA from 5 μg of poly(A)$^+$RNA was synthesized with oligo(dT) and random hexamer primers mixture and cloned into the λgt11 vector using SuperscriptII choice system (Gibco BRL, Life Technologies). We obtained 6×10^6 independent λphage clones.

cDNA cloning and sequencing

We screened 22-26 hpf zebrafish embryo cDNA libraries as described in Sambrook and Russell (Sambrook and Russell, 2001) using 582 bp PCR fragment of D204 that was identified by ODD comparing wild-type and LIM^{Isl-3}-overexpressing embryos (Hirate et al., 2001). Seventeen independent positive clones were isolated from 10^6 recombinant clones. Restriction fragments of cDNA were cloned into pBluescriptII (Stratagene), and sequenced by using Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech) and BigDye terminator cycle sequencing kit (Applied Biosystems). Sequences were determined by GENETYX-MAC 9.01 program (Software Development). The Accession Number for the zebrafish *plexinA4* cDNA is AB103158. Homology searches were performed using BLAST algorithm at the NCBI (Altschul et al., 1990). The phylogenic tree was constructed using CLUSTAL W multiple sequence alignment program at the GenomeNet (http://clustalw.genome.ad.jp/). Mouse (Kameyama et al., 1996a; Kameyama et al., 1996b; Suto et al., 2003) and *Xenopus* (Ohta et al., 1995) PlexinA family were used for comparison.

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was performed according to the standard method (Westerfield, 2000). The 1560 bp fragment of *plexinA4* cDNA that includes 580 bp of 3′ untranslated region (UTR) was used as a template for synthesizing the antisense RNA probe. Antisense RNA probe was synthesized using the digoxigenin-RNA labeling kit (Roche Diagnostics). The immunohistochemistry and double staining were performed as described previously (Segawa et al., 2001; Yeo et al., 2001). The dilution rate of primary antibodies was 1:2000 for the anti-acetylated α-tubulin antibody (Sigma), 1:500 for anti-GFP antibody (Santa Cruz Biotechnology) and 1:500 for 3A10 monoclonal antibody (Developmental Studies Hybridoma Bank at the University of Iowa).

Plasmids construction

The plasmids for generating transgenic zebrafish of dnPlexinA4 (pSS-hsp70:dnPlexinA4-GFP) or PlexinA4 (pSS-hsp70:PlexinA4-GFP) were constructed in the following manner. The backbone plasmid was pBluescript II SK. The *Nhe*I-*Ssp*I fragment of pIRES2-EGFP vector (Clontech) was inserted between the *Xba*I and filled-in *Not*I sites of pBluescript II SK to create pSK-IRES2-EGFP. The *Eco*RI-*Pst*I fragment of the zebrafish *heat-shock protein 70* (*hsp70*) promoter (Halloran et al., 2000) was inserted between the *Eco*RI and *Pst*I sites of pSK-IRES2-EGFP to create pHsp-IRES2-EGFP. Then, the *Xho*I-*Eco*RI fragment of the primary sensory neuron-specific enhancer (SS) of the zebrafish *islet1* gene (Higashijima et al., 2000) was inserted between the *Xho*I and *Eco*RI sites of pHsp70:IRES2-EGFP to create pSS-hsp70:IRES2-EGFP.

The 2280 bp *Bam*HI-*Xba*I fragment that contains the extracellular region of *plexinA4* was inserted between the *Bam*HI and *Xba*I sites of pCS2+ vector (Turner and Weintraub, 1994). The 1604 bp *Bam*HI fragment that contains short 5′UTR, the first ATG and the extracellular region of *plexinA4* were inserted to the *Bam*HI site of this plasmid to

create pCS2+dnPlexinA4. The *Hin*dIII site of the *Hin*dIII-*Sna*BI fragment of pCS2+dnPlexinA4 was filled-in by Klenow fragment of DNA polymerase, and this fragment was inserted into the *Sma*I site of pSS-hsp70:IRES2-EGFP to create pSS-hsp70:dnPlexinA4-IRES2-EGFP.

To construct recombinant plasmids encoding the chimeric proteins of PlexinA4 and dnPlexinA4 fused with green fluorescent proteins (GFP), polymerase chain reaction (PCR) amplifications of the 3′ region of PlexinA4 and dnPlexinA4 were performed using *plexinA4* cDNA as a template, respectively, with the following sets of oligonucleotide primers; PlexA4-5′, 5′-TAT ACT ACC CAA ATC CTG TG-3′; and dnPlexA4-3′, 5′-CTT GCC CAT GGA GGC AAT TAA GA-3′ or PlexinA4-3′, 5′-AAA TGT GGC CAT GGG CTC TC-3′ (underlines indicate the *Nco*I sites for ligation with GFP cDNA fragment at its first ATG). The amplified DNA fragment was subcloned into pGEM-T Easy vector (Promega) to generate pGEM-3′dnPlex and pGEM-3′Plex. The *Sac*I-*Sac*II fragment of pSS-hsp70:dnPlexinA4-IRES2-EGFP was inserted between the *Sac*I and *Sac*II sites of pBluescriptII KS, and the *Sac*I-*Nco*I fragment of pGEM-3′dnPlex was subcloned between the *Sac*I and *Nco*I sites of this plasmid. This plasmid contains the C-terminal region of PlexinA4 fused with GFP in the *Pma*CI-*Not*I fragment. This *Pma*CI-*Not*I fragment was subcloned between the *Pma*CI and *Not*I sites of pSS-hsp70:dnPlexinA4-IRES2-EGFP to create pSS-hsp70:dnPlexinA4-GFP. The *Xba*I-*Nco*I fragment of pGEM-3′Plex was inserted between the *Xba*I and *Nco*I sites of pSS-hsp70:dnPlexinA4-GFP to create pSS-hsp70:PlexinA4-GFP.

The plasmid for single cell imaging (pSSICP-Kaede) was constructed by replacing the GFP cDNA of SSICP-GFP (Higashijima et al., 2000) with Kaede cDNA (kindly provided by Dr A. Miyawaki, Brain Science Institute, Riken, Japan) (Ando et al., 2002).

Generation of the transgenic zebrafish

Constructed plasmids were purified using QIAprep Spin Miniprep kit (QIAGEN). DNA solution of 20 μg/ml in distilled water was microinjected into one- to four-cell stage zebrafish embryos. The injected embryos were raised to sexual maturity, and crossed with each other to identify the founder fish pairs that bear the progeny transgenic for pSS-hsp70:dnPlexinA4-GFP. Transgenic embryos were identified by their expression of the GFP fluorescence in the trigeminal sensory ganglion neurons and Rohon-Beard neurons that was induced by sensory neuron-specific enhancer of the zebrafish *Islet1* and in the whole body following the heat shock treatment at 39°C for 45 minutes. Two lines, termed *Tg(SS-hsp70:dnPlexinA4-GFP)*^{rw016a} and *Tg(SS-hsp70:dnPlexinA4-GFP)*^{rw016b}, in which dnPlexinA4-GFP fusion protein was induced, were used for further analysis.

Injection of morpholino oligonucleotide

Specific 25-base antisense morpholino oligonucleotide (AMO) (Gene Tools) against *plexinA4* was designed to contain the first start codon (ATG), and control morpholino oligonucleotide (MO) with four-base mismatch was designed. Their sequences were as follows: AMO against *plexinA4* mRNA, 5′-TCCAT CTCCT ATTGT GAAAA GCCAT-3′; control MO, 5′-TCCtT CTCgT ATTGT GAAtA GCgAT-3′ (where the mismatch bases are indicated by lowercase letters). They were dissolved in Danieou buffer as instructed by Nasevicius and Ekker (Nasevicius and Ekker, 2000) at the concentration of 2 mg/ml and microinjected by air pressure into the one- to four-cell stage zebrafish embryos.

Heat shock treatment of transgenic zebrafish

Heterozygous embryos of transgenic line *Tg(hsp70:Slit2-GFP)*^{rw015d} (previously called HS2E-4S) (Yeo et al., 2001) were used for Slit2-GFP-overexpression experiments. Double transgenic zebrafish embryos, which can overexpress both Slit2-GFP and dnPlexinA4-GFP, were obtained from crossing *Tg(hsp70:Slit2-GFP)*^{rw015d} homozygous with the *Tg(SS-hsp70:dnPlexinA4-GFP)*^{rw016b} homozygous fish. These embryos were maintained at 39°C for 45 minutes to induce the transgene expression at 14 hpf.

Time-lapse observation of axonal outgrowth of a single primary sensory neuron expressing Kaede

A part of the embryonic spinal cord expressing Kaede in Rohon-Beard neurons was exposed to UV light using the 40× water immersion objective lens (ACHROPLAN 40X/0.80w, Carl Zeiss) and the filter set 01 (BP 365/12, FT 395, LP 397. Carl Zeiss) of Zeiss LSM510 until the emitted light turns from pale blue to red. Confocal images were captured from 23 hpf and 27 hpf embryos using the same microscope.

Scoring branching of the peripheral axons of Rohon-Beard neurons

Embryos were stained with anti-acetylated α-tubulin antibody (Sigma). Camera lucida drawings of the peripheral axons of Rohon-Beard neurons were made. We divided the trunk region into six longitudinal areas along the dorsoventral axis as shown in Fig. 4G, and counted the number of branching points of the peripheral axons of Rohon-Beard neurons in each region caudal to the transition between the yolk and yolk extension.

Results

Overexpression of the LIM domains of the Islet2 reduces expression level of D204 mRNA

As already mentioned, the expression of D204 mRNA in the tectum is almost completely abolished in the LIM^{Isl-3}-overexpressing embryos (Fig. 1A,A′) (Hirate et al., 2001). *Islet2* is expressed in the same primary sensory neurons that express D204 mRNA (Tokumoto et al., 1995), and overexpression of LIM^{Isl-2} eliminates the peripheral axon extension by the Islet2-positive sensory neurons, i.e. the trigeminal sensory ganglion neurons (Fig. 1B,B′) and Rohon-Beard neurons (Fig. 1D,D′) (Segawa et al., 2001). In the LIM^{Isl-2}-overexpressing embryos, expression of D204 mRNA in the trigeminal sensory ganglion neurons (Fig. 1C,C′) and Rohon-Beard neurons (Fig. 1E,E′) was significantly reduced. But its expression in the hindbrain remained intact as in wild-type embryos (Fig. 1C,C′).

Molecular cloning of full-length D204 revealed that it encodes the zebrafish ortholog of PlexinA4

Sequence analysis of a full-length cDNA fragment for D204 and subsequent BLAST homology search revealed that D204 encodes a member of PlexinA family. Phylogenic tree analysis among mouse PlexinA family, *Xenopus* Plexin and zebrafish PlexinA revealed that D204 encodes the zebrafish ortholog of PlexinA4 (Fig. 2A) (Suto et al., 2003). Proteins of PlexinA family consist of a Sema domain, Met related sequence/Plexin, semaphorin, integrin domain (MRS/PSI domain), glycine-proline rich/immunoglobin-like fold shared by plexins and transcription factors motif (G-P/IPT motif) and intracellular Plexin family conserved Sex Plexin domain (SP domain) (Bork et al., 1999). The overall identity of zebrafish PlexinA4 in the amino acid sequence to mouse PlexinA4 is 67.6%. The extracellular region shows an average of 59.5% identity with Sema domain, MRS/PSI domain and G-P/IPT motif, showing 60.4%, 61.5% and 55.9% identity, respectively. The intracellular region motif shows an average of 83.5% identity with three conserved domains, the domain 1-3, respectively, showing 85.5%, 83.3% and 81.8% identity (Fig. 2B).

plexinA4 is expressed in specific subtypes of neurons in the brain

Double staining by immunohistochemistry with anti-acetylated α-tubulin antibody for the initial axonal scaffold of the embryonic brain and in situ hybridization for *plexinA4* mRNA revealed that *plexinA4* mRNA is expressed in the tectal region anterior to the MHB, the hindbrain, the neurons in the nucleus of the anterior commissure (nAC), the nucleus of postoptic commissure (nPOC), the nucleus of posterior commissure (nPC), the nucleus of medial lateral fascicle (nMLF), and the epiphysis (ep) (Fig. 3A) (Chitnis and Kuwada, 1990; Wilson et al., 1990). In the hindbrain, *plexinA4* is expressed in a segmental manner (Fig. 3B). Double staining against GFP protein and *plexinA4* mRNA in a 22-hpf *Tg(α-actin:GFP)* transgenic embryo which expresses GFP in Mauthner neurons and the segmentally distributed reticulospinal neurons in the other rhombomeres of the hindbrain (Fig. 3B,C) (Higashijima et al., 1997) (H.W. and H.O., unpublished) revealed that *plexinA4* is expressed in these neurons.

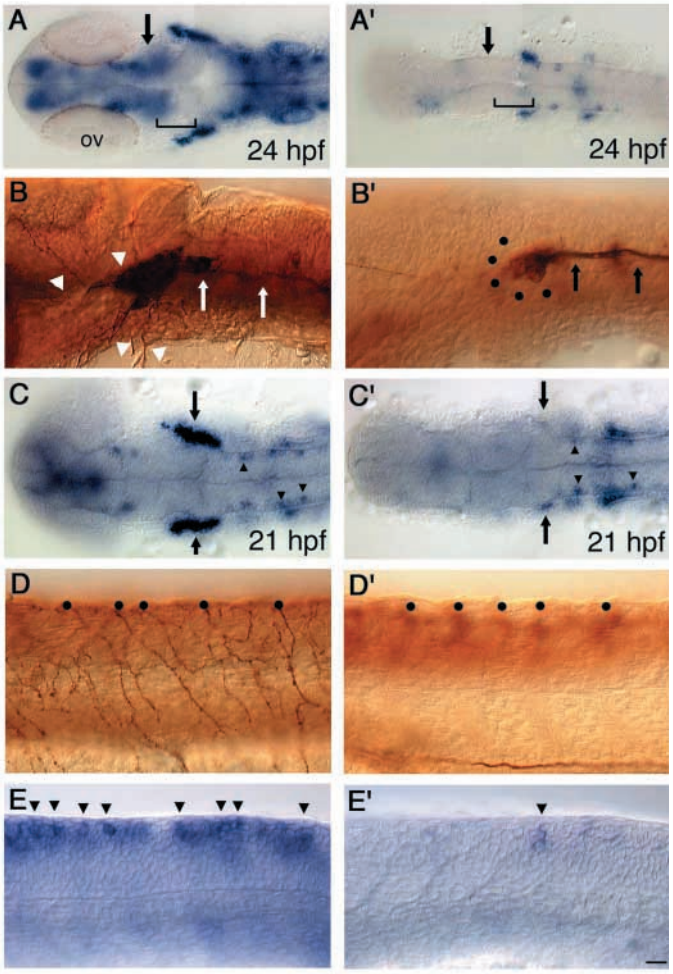

Fig. 1. D204 is a candidate downstream gene of Islet3 and Islet2. (A,A′) D204 mRNA expression in 24 hpf normal (A) and LIM^{Isl-3}-overexpressing (A′) zebrafish embryos. The expression in tectum was reduced in LIM^{Isl-3}-overexpressing embryo (arrows in A and A′). The embryos overexpressing LIM^{Isl-3} lack optic vesicles (ov) and MHB (brackets). (B,B′,D,D′) The peripheral axons of both the trigeminal sensory ganglion neurons (arrowheads in B and black circles in B′) and Rohon-Beard neurons (black circles in D and D′) were eliminated by overexpression of LIM^{Isl-2} (B′,D′), while their central axons (arrows in B and B′) remained intact as in normal embryos at 24 hpf. The axons were stained by anti-acetylated α-tubulin antibody. Embryos in B and D are normal controls. (C,C′,E,E′) D204 expression in the trigeminal sensory ganglion neurons (arrows) and Rohon-Beard neurons (arrowheads) were reduced in LIM^{Isl-2}-overexpressing 21 hpf embryos (C′,E′), while the expression of D204 in the hindbrain remained intact (arrowheads in C′). Embryos in C and E are normal controls. (A,A′,C,C′) Dorsal views; anterior leftwards. (B,B′,D,D′,E,E′) Lateral views; anterior leftwards; dorsal towards the top. Scale bar: 20 μm in A,C; 10 μm in B,D,E.

GFP fusion proteins of both PlexinA4 and dnPlexinA4 are localized to both central and peripheral axons of the primary sensory neurons

The LIM^{Isl-2}-overexpressing embryos show specific defect in the peripheral axon extension by the trigeminal sensory ganglion neurons and Rohon-Beard neurons (Segawa et al., 2001; Fig. 1B′,D′) and reduced expression of *plexinA4* mRNA in these neurons (Fig. 1C′,E′), suggesting that PlexinA4 may play an important role in the peripheral axon outgrowth. If it is the case, PlexinA4 may function by being selectively localized in the peripheral axons of both the trigeminal sensory ganglion neurons and Rohon-Beard neurons.

We transiently expressed PlexinA4 fused with green fluorescent protein (GFP) at the C terminus (PlexinA4-GFP),

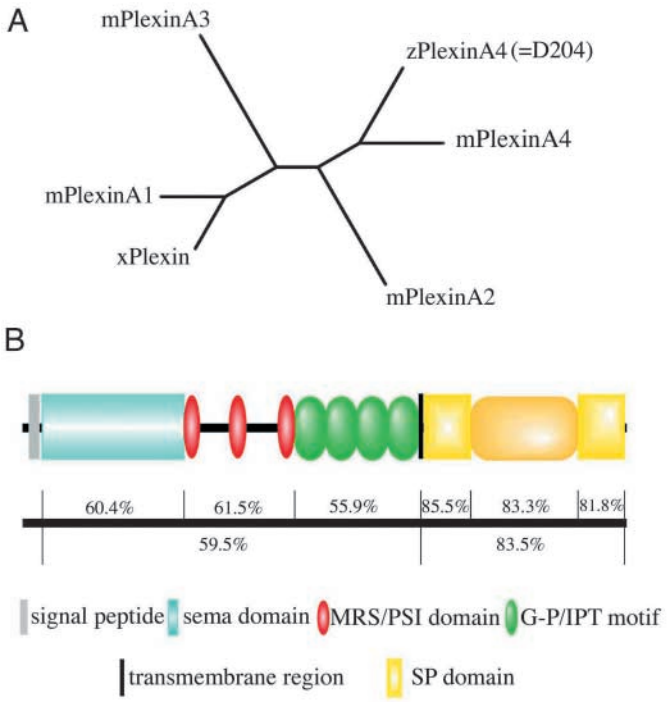

Fig. 2. D204 encodes the zebrafish ortholog of PlexinA4 (Accession Number, AB103158). (A) The phylogenic tree indicates that D204 encodes the zebrafish ortholog of PlexinA4. (B) Schematic drawing of the domain structure of the Plexin family and identity of amino acids between zebrafish and mouse PlexinA4. MRS/PSI domain is Met related sequence/plexin, semaphorin, integrin domain. G-P/IPT motif is glycine-proline rich/immunoglobin-like fold shared by plexins and transcription factors motif. SP domain is plexin-specific sex plexin domain. m, mouse; x, *Xenopus laevis*; z, zebrafish.

in both the trigeminal sensory ganglion neurons and Rohon-Beard neurons by injecting a plasmid, pSS-hsp70:PlexinA4-GFP (Fig. 3D), into the one-cell stage embryos, which drives expression of PlexinA4-GFP fusion protein under control of the primary sensory neuron-specific enhancer (SS) of the zebrafish *islet1* gene at room temperature (Higashijima et al., 2000). The PlexinA4-GFP fusion protein is distributed both in the central and peripheral axons of both the trigeminal sensory ganglion neurons (Fig. 3E) and Rohon-Beard neurons (Fig. 3F).

PlexinA4 protein lacking the cytoplasmic region is known to act as a dominant-negative variant (Takahashi et al., 1999; Rohm et al., 2000). A stable zebrafish line *Tg(SS-hsp70:dnPlexinA4-GFP)* transgenic for pSS-hsp70:dnPlexinA4-GFP driving expression of this dominant-negative variant fused with GFP (dnPlexinA4-GFP) under control of the same primary sensory neuron-specific enhancer was also created (Fig. 3G). In the embryos of this line, dnPlexinA4-GFP fusion protein was expressed both in the trigeminal sensory ganglion neurons (Fig. 3H) and Rohon-Beard neurons (Fig. 3I), and they were also distributed both in the central and peripheral axons.

PlexinA4 promotes branching in the peripheral axons of Rohon-Beard neurons

As the promoter of the zebrafish *heat-shock protein 70* (*hsp70*) gene was used as a core promoter for creating these transgenic fish, heat shock treatment can induce ubiquitous overexpression of dnPlexinA4-GFP in addition to the basal expression specifically observed in the primary sensory neurons (Halloran et al., 2000) (Fig. 3J). Specific AMO (Nasevicius and Ekker, 2000) was designed for *plexinA4* mRNA. We checked its activity by examining whether it can prevent the translation of dnPlexinA4-GFP fusion protein after heat shock treatment in this transgenic embryo when AMO was injected at the one-cell stage. In transgenic embryos, heat-

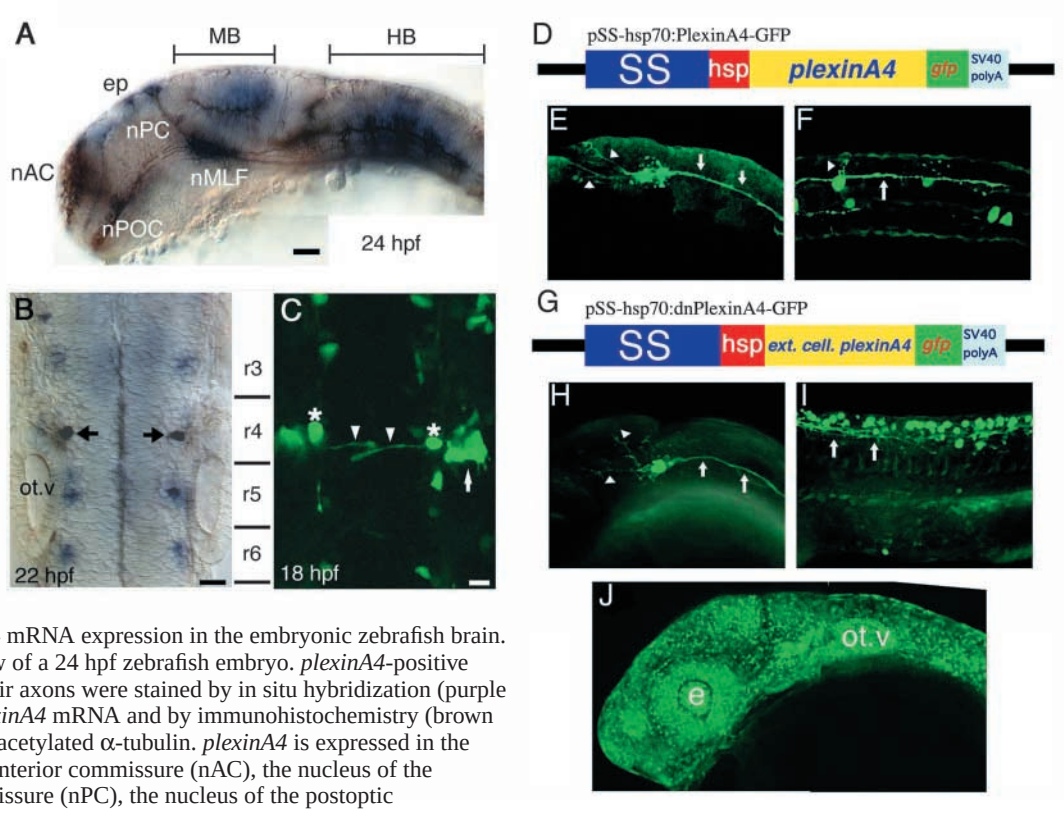

Fig. 3. *plexinA4* mRNA expression in the embryonic zebrafish brain. (A) Lateral view of a 24 hpf zebrafish embryo. *plexinA4*-positive neurons and their axons were stained by in situ hybridization (purple signals) for *plexinA4* mRNA and by immunohistochemistry (brown signals) for the acetylated α-tubulin. *plexinA4* is expressed in the nucleus of the anterior commissure (nAC), the nucleus of the posterior commissure (nPC), the nucleus of the postoptic commissure (nPOC), the epiphysis (ep), the nucleus of the medial lateral fascicle (nMLF), the tectum and the hindbrain. MB, midbrain; HB, hindbrain; anterior, leftwards; dorsal towards the top. (B) Dorsal view of a 22 hpf *Tg(α-actin:GFP)* transgenic embryo which expresses GFP (brown signals) in Mauthner neurons (arrows) in the hindbrain. *plexinA4* expression (purple signals) is detected in GFP-positive Mauthner neurons (arrows) in the rhombomere 4 (r4) and the segmentally distributed reticulospinal neurons in the other rhombomeres. (C) Dorsal view of an 18 hpf *Tg(α-actin:GFP)* transgenic embryo that expresses GFP in Mauthner neurons (asterisks). White arrowheads indicate the axons from Mauthner neurons and a white arrow indicates 8th neurons. r3-r6, rhombomere 3 to rhombomere 6; ot.v, otic vesicles; anterior is towards the top. (D-I) Cellular localization of the transiently expressed PlexinA4-GFP and dnPlexinA4-GFP fusion proteins in the primary sensory neurons. To visualize the cellular localization of both PlexinA4-GFP and dnPlexinA4-GFP in the primary sensory neurons, we injected plasmids (D,G) that can induce expression of PlexinA4-GFP and dnPlexinA4-GFP fusion protein under control of the primary sensory neuron-specific enhancer (SS) of the zebrafish *isl1* gene. In the trigeminal sensory ganglion neurons (E,H) and Rohon-Beard neurons (F,I), both PlexinA4-GFP (E,F) and dnPlexinA4-GFP (H,I) fusion protein were localized in both of the central (arrows in E,F,H,I) and peripheral (arrowheads in E,F,H) axons. The stable transgenic zebrafish line that carries pSS-hsp70:dnPlexinA4-GFP (G) was established. In this transgenic zebrafish [*Tg(SS-hsp70:dnPlexinA4-GFP)*], we can induce expression of dnPlexinA4-GFP fusion protein ubiquitously by heat-shock treatment (J). e, eye; ot.v, otic vesicles; SS, sensory neuron-specific enhancer of the zebrafish *isl1* gene; hsp, zebrafish *heat-shock protein 70* (*hsp70*) promoter; *gfp*, green fluorescence protein; SV40 polyA, SV40 late polyadenylation site. (E,F,H-J) Lateral view; anterior is leftwards; dorsal is towards the top. Scale bars: 20 μm in A; 10 μm in B and C.

induced dnPlexinA4-GFP signals were detected, and signals of in situ hybridization for *gfp* were also detected ubiquitously. However, in AMO-injected transgenic embryos, no GFP signals were detected after heat-shock treatment. But the *gfp* mRNA was ubiquitously expressed similarly as in uninjected embryos (data not shown). These results justified our use of this AMO for the analysis of loss-of-function phenotypes of *plexinA4*.

Staining with anti-acetylated α-tubulin antibody revealed that, in the AMO-injected embryos, the number of the peripheral axons of Rohon-Beard neurons was reduced as demonstrated in the lateral view (Fig. 4B,B′). However, as revealed by the dorsal view (Fig. 4E), the number of the main trunks of the peripheral axons of Rohon-Beard neurons (arrowheads) exiting the spinal cord was similar as in the normal embryos (Fig. 4D) and in the embryos injected with the control MO (Fig. 4F). This phenotype caused by injection of AMO partially recapitulated the defects observed in the embryos that were injected with mRNA for $LIM^{Isl\text{-}2}$, although the peripheral axons of Rohon-Beard neurons were completely eliminated in the embryos overexpressing $LIM^{Isl\text{-}2}$ (Fig. 1D′).

To analyze the phenotype more precisely, we made the camera lucida drawing and counted the number of the branch termini and branching points of the peripheral axons (Fig. 4A′-C′) distributed in each subdivision of the trunk along the dorsoventral axis (D1-D3,V3-V1) (Fig. 4G).

The number of peripheral branches was reduced to about 67.5% compared with wild-type embryos and with the embryos injected with control MO. The dorsoventral distribution of branch termini of the peripheral axons showed slight increase in shorter axons (data not shown). Most conspicuously, the number of branching points was prominently reduced to 42% in the AMO-injected embryos (n=24) (Fig. 4H), especially in the dorsal region (D2 region) where the peripheral axon branches elaborate most extensively in the wild-type embryo (n=8), and in the embryo injected with control MO (n=13) (Fig. 4I).

To further confirm our observation at the single-cell level, we carried out time-lapse observation of the growth of a single Rohon-Beard neuron in the embryo ubiquitously overexpressing Slit2-GFP by expressing Kaede in this neuron (Ando et al., 2002) (Fig. 5A,B,D,E). Kaede emits much brighter red signal than DsRed2 in zebrafish embryos when it is photoconverted by exposure to UV light. The plasmid (pSSICP-Kaede) which can drive expression of Kaede fluorescent protein under control of the sensory neuron-specific enhancer of *isl1* (SS) was injected into the one-cell stage normal embryos with AMO against *plexinA4*. A part of the

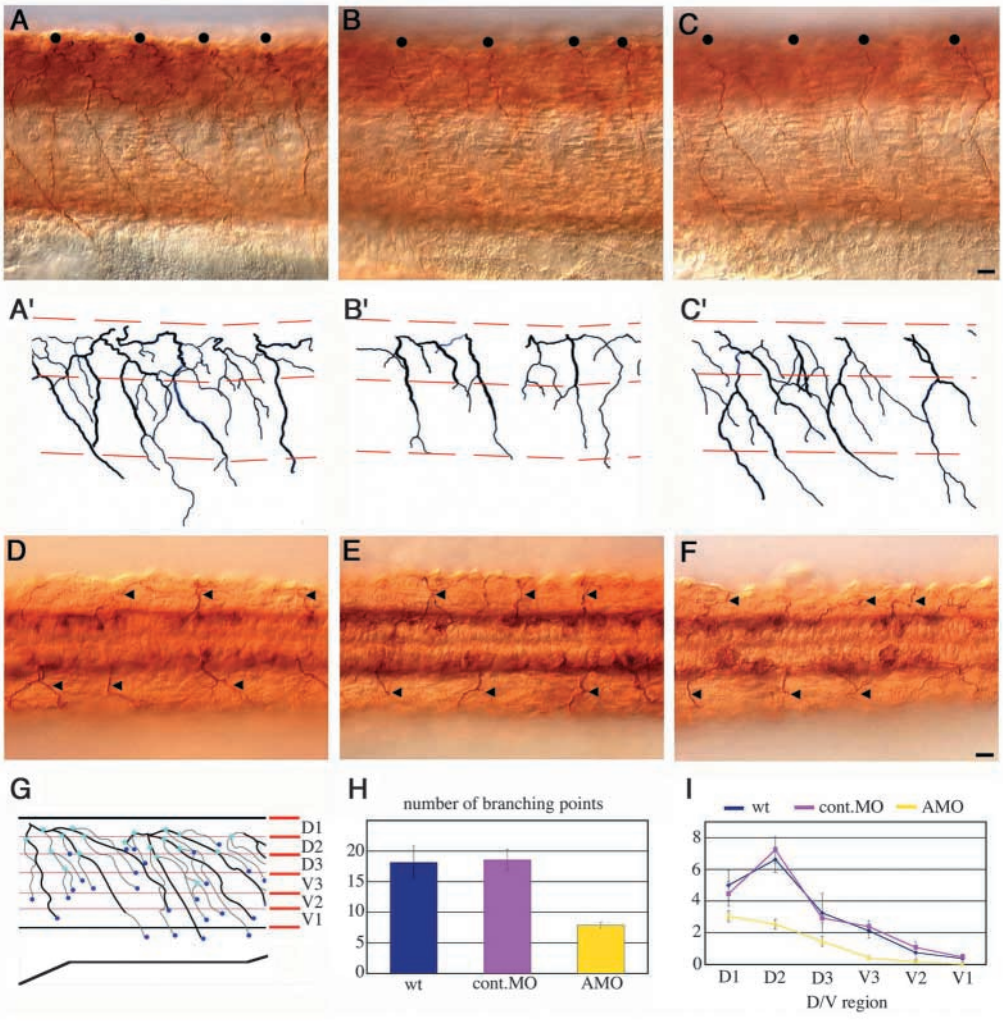

Fig. 4. Inhibition of PlexinA4 translation by injection of AMO against *plexinA4* mRNA caused reduction in the number of branches of the peripheral axons of Rohon-Beard neurons. (A-F) The peripheral axons of Rohon-Beard neurons (black circles) of 24 hpf embryos were visualized by anti-acetylated α-tubulin antibody staining (A-F), and camera lucida drawings of them were made (A′-C′). The AMO-injected embryos showed reduction in the number of the peripheral axons of Rohon-Beard neurons, as shown in the lateral view (B,B′) compared with normal (A,A′) and control MO-injected embryos (C,C′). But in the dorsal view (E), a similar number of main trunks of the peripheral axons (arrowheads) extended out of the spinal cord as in the normal (D) and control MO-injected (F) embryos. (A-C,A′-C′) Lateral view; anterior is leftwards; dorsal is towards the top. (D-F) Dorsal view; anterior is leftwards. Scale bars: in C, 10 μm for A-C; in F, 10 μm for D-F. (G-I) We subdivided the trunk region of the embryos into six longitudinal areas along the dorsoventral axis (D1-D3, V3-V1), and counted the number of branching points of the peripheral axons of Rohon-Beard neurons stained by anti-acetylated α-tubulin antibody (G). In AMO-injected embryos, the number of the branching points was prominently reduced (H), especially in the D2 region (I).

embryonic spinal cord expressing Kaede in Rohon-Beard neurons was exposed to UV light until the emitted light turns from pale blue to red. We confirmed the reduction in the number of branching points of peripheral axons of a single Rohon-Beard neuron in the AMO-injected embryo (*n*=3).

We also confirmed that the similar phenotype could be observed in the transgenic embryos overexpressing dnPlexinA4-GFP fusion protein specifically in Rohon-Beard neurons, which was induced by the primary sensory neuron-specific enhancer (SS) of the zebrafish *isl1* gene (Fig. 6A,B). The number of branching points was reduced to 42.3%, especially in the D2 region, in the transgenic embryos that express dnPlexinA4-GFP in the Rohon-Beard neurons (*n*=20) when compared with normal embryos (*n*=10) (Fig. 6C,D).

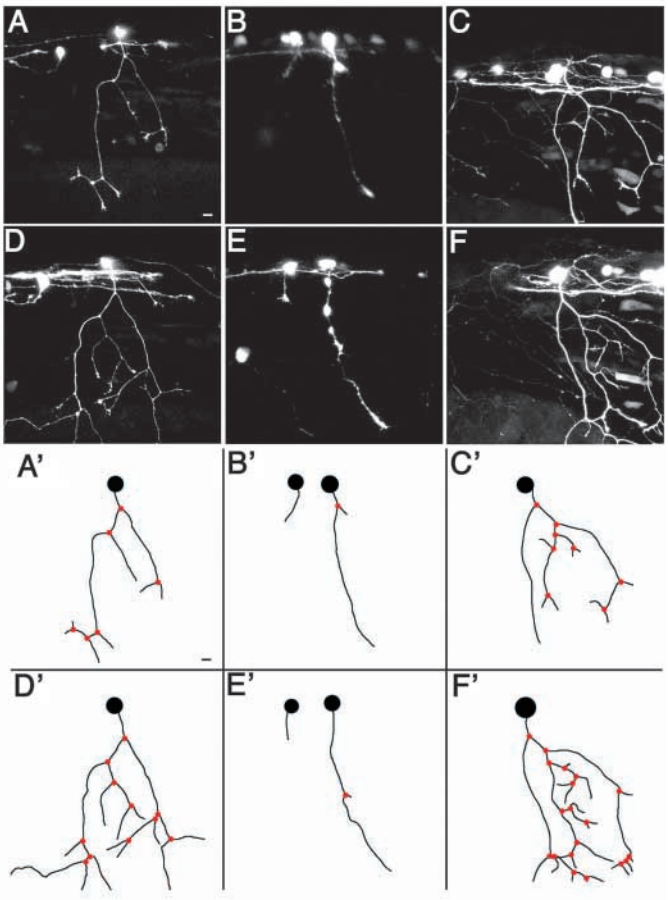

Fig. 5. Time-lapse observation of Rohon-Beard neurons that were separately labeled with Kaede in the embryos with a reduced level of PlexinA4 expression or with ubiquitous overexpression of Slit2. Stacked confocal images of a single Rohon-Beard neuron expressing Kaede transiently under control of the primary sensory neuron-specific enhancer (SS) of the zebrafish *isl1* gene (A-F). In comparison with normal embryo (A,D,A′,D′), the number of branching points of peripheral axons was reduced in the embryo injected with AMO against *plexinA4* (B,E,B′,E′). However, the number of branching points increased in the Slit2-overexpressing embryo (C,F,C′,F′). (A-F) Confocal images; lateral view; anterior leftwards; dorsal towards the top. (A-C) 23 hpf embryos. (D-F) The same embryos as shown in A-C at 27 hpf. (A′-F′) Schematic drawings of A-F in which cell bodies and branching points of the peripheral axons were indicated by black circles and red dots, respectively. Scale bar: 10 μm.

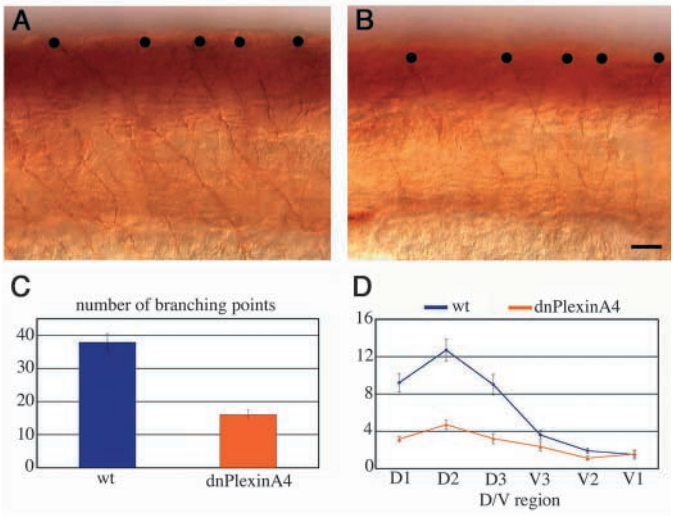

Fig. 6. Transgenic overexpression of dnPlexinA4-GFP in the Rohon-Beard neurons caused reduction in the number of branching of the peripheral axons of Rohon-Beard neurons. Anti-acetylated α-tubulin antibody staining of 26 hpf embryos of the transgenic zebrafish (B), which expresses dnPlexinA4-GFP fusion protein in the Rohon-Beard neurons (black circles), showed significant reduction in the number of branching points of the peripheral axons compared with that in the normal 26 hpf embryo (A,C), especially in the D2 region (D). Lateral view; anterior is leftwards; dorsal is towards the top. Scale bar: 10 μm.

In contrast to the effect on the primary sensory neurons, injection of AMO against *plexinA4* mRNA and ubiquitous overexpression of dnPlexinA4-GFP fusion protein did not impair the axonal outgrowth by the primary motoneurons, which do not express *plexinA4* normally (data not shown).

PlexinA4 is necessary for the Slit signaling in promotion of sensory axon branching

Slit plays a crucial role in axon pathfinding (Brose et al., 1999; Li et al., 1999; Wang et al., 1999; Guthrie, 1999; Brose and Tessier-Lavigne, 2000). In zebrafish, ubiquitous overexpression of Slit2 causes abnormal axonal pathfinding in the axons of the trigeminal sensory ganglion neurons and axons of Mauthner neurons, excessive branching of the peripheral axons of the trigeminal sensory ganglion neurons and Rohon-Beard neurons, and defasciculation of the medial longitudinal fascicles (Yeo et al., 2004) (see also Fig. 7B,F). We showed that Robo2 is specifically expressed in the trigeminal sensory neurons, and disruption of this gene in the mutant embryos cancels the branch promotion effect of excessive Slit2 overexpression (Yeo et al., 2004). Robo3 is reported to be expressed in the dorsal spinal cord of zebrafish embryos (Lee et al., 2001).

The trigeminal sensory ganglion neurons and Rohon-Beard neurons also express *plexinA4*, as shown in Fig. 1C,E. The abnormal increase in the number of the peripheral axon branches of Rohon-Beard neurons in the Slit2-overexpressing embryos [*n*=19, ~180% increase compared with normal embryos (*n*=10)] [Fig. 7B,L,M, see also Fig. 5C,F (*n*=3)] was opposite to the phenotype induced by loss of function of PlexinA4, as described above (Fig. 4, Fig. 6 and Fig. 5B,E). Therefore, we examined whether PlexinA4 and Slit2 might have functional interaction with each other.

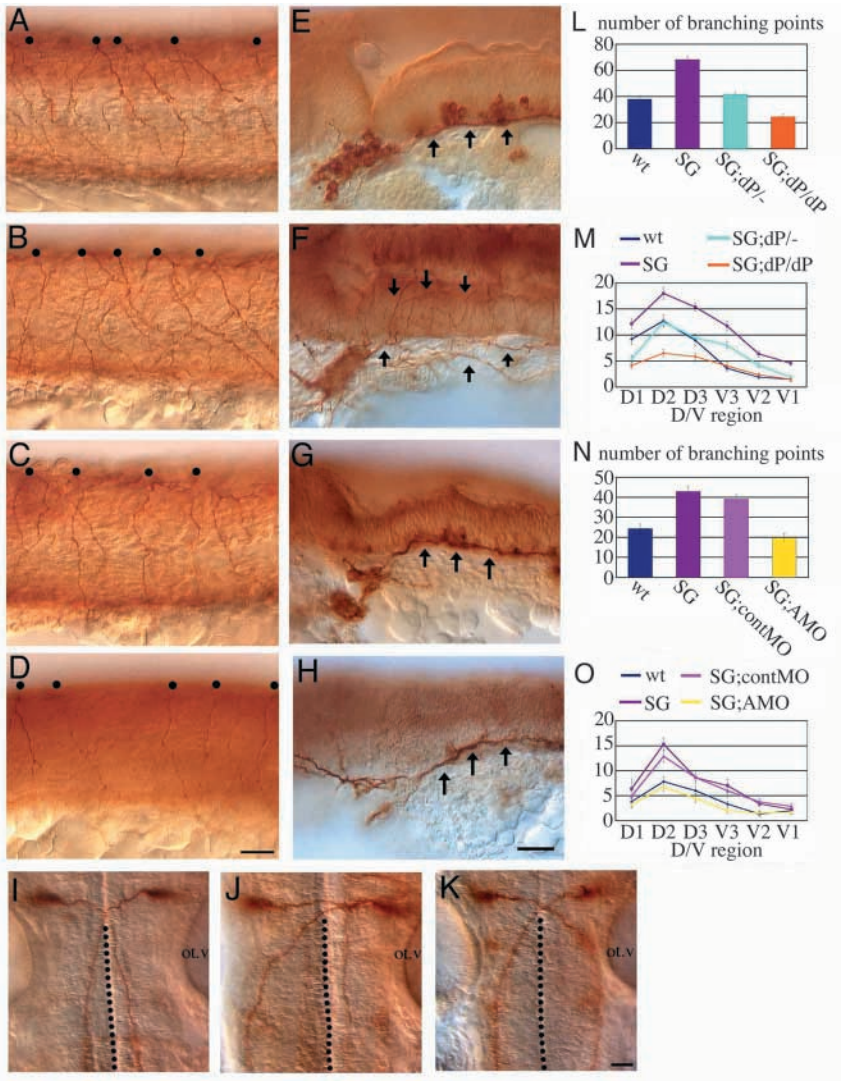

Fig. 7. Loss of function of PlexinA4 rescued the Slit2 overexpression phenotype in the primary sensory neurons. Ubiquitously overexpressed Slit2 induced excessive branching in the peripheral axons of Rohon-Beard neurons (black circles) (B) compared with branching in normal embryos (A). Simultaneous overexpression of Slit2 with dnPlexinA4 (C) and with injection of the AMO against PlexinA4 (D) rescued this phenotype. Overexpression of Slit2 before the central axons of trigeminal sensory ganglion neurons (arrows) enter the hindbrain prevents these axons from entering the hindbrain and induce abnormal defasciculation (F). The embryo in E is a normal control. In 40% of transgenic zebrafish that carry the transgenes for both *Slit2* and *dnPlexinA4* under control of the *hsp70* promoter (G), failure of the central axons to enter the hindbrain by Slit2-overexpression was rescued. In addition, the same defects were also rescued in 44% of the AMO-injected embryos (H). (A-D) Lateral view; anterior is leftwards; dorsal is towards the top. (E-H) Dorsolateral oblique view; anterior is leftwards. Scale bars: in D, 20 μm for A-D; in H, 20 μm for E-H. Repression of PlexinA4 function could not rescue the abnormal axonal pathfinding of Mauthner neurons induced by Slit2 overexpression. (I) The axons from Mauthner neurons of the normal embryo immunostained with 3A10 antibody cross the midline (broken line) and extend posteriorly near the midline. (J) In the Slit2-overexpressing embryo, the axons of Mauthner neurons take irregular trajectories. (K) Simultaneous overexpression of dnPlexinA4-GFP could not rescue this phenotype. ot.v, otic vesicle. Scale bar: 10 μm. Induction of excessive branching of the peripheral axons of Rohon-Beard neurons observed in the Slit2-overexpressing embryos (SG) was canceled in the embryos overexpressing both Slit2 and dnPlexinA4-GFP in a dose-dependent manner. (L,M) The number of branching points was reduced more severely in the embryos carrying the dnPlexinA4-GFP transgene homozygously (SG;dP/dP) than in the heterozygous embryos (SG;dP/–). (N,O) Injection of the AMO against PlexinA4 (SG;AMO) also rescued the excessive branching of the peripheral axons of Rohon-Beard neurons induced by Slit2 overexpression (SG), whereas injection of control-MO (SG;contMO) could not rescue this phenotype. wt, wild-type embryos.

To examine the relationship between Slit2 and PlexinA4, we made transgenic zebrafish which could overexpress both Slit2-GFP and dnPlexinA4-GFP under control of the *hsp70* promoter (Yeo et al., 2001). In these embryos, the excessive branching of the peripheral axons of Rohon-Beard neurons which would be induced by overexpression of Slit2-GFP alone (Fig. 7B) was not observed (Fig. 7C). This effect of dnPlexinA4 expression is dose dependent. The embryos that carry SS-hsp70:dnPlexinA4-GFP transgene homozygously showed more prominent reduction in the number of the branching points (n=20, 64.4%) than did the embryos carrying the transgene heterozygously (n=20, 109.5%) (Fig. 7L,M). In addition, when we injected AMO against *plexinA4* into the transgenic embryo overexpressing Slit2-GFP, the excessive branching was also canceled (n=8, 82%) (Fig. 7D), but the Slit2-overexpressing embryo which was injected with control-MO (n=8, 160%) showed similar excessive branching to the uninjected embryos (n=8, 176.5%) (Fig. 7N,O).

In normal embryos, the central axons of the trigeminal sensory ganglion neurons project into the hindbrain as a tight fascicle (Fig. 7E). Our recent study revealed that these central axons are extensively defasciculated and fail to enter the hindbrain in the embryos ubiquitously overexpressing Slit2-GFP under control of the *hsp70* promoter (Fig. 7F) (Yeo et al., 2004). By contrast, in 40% of embryos transgenically overexpressing both Slit2-GFP and dnPlexinA4-GFP under control of the *hsp70* promoter, the central axons were prevented from defasciculation and entered the hindbrain as in normal embryos (Fig. 7G; Table 1). Similar results were obtained in 44% of the embryos overexpressing Slit2-GFP when they were also injected with AMO for *plexinA4* (Fig. 7H; Table 1).

The axons of Mauthner neurons cross the midline and extend caudally on the contralateral side (Fig. 7I). In Slit2-overexpressing embryos, Mauthner axons show abnormal projection patterns. Some axons descend more laterally, or the

Table 1. Projection patterns of the central axons of the trigeminal sensory ganglion neurons

Construct being overexpressed	Out*	In*	Total
Slit2-GFP	20 (100%)	0 (0%)	20 (100%)
Slit2-GFP and dnPlexinA4-GFP	60 (60%)	40 (40%)	100 (100%)
Slit2-GFP and AMO	28 (56%)	22 (44%)	50 (100%)

*The number of the embryos in which the central axons of the trigeminal sensory ganglion neurons failed to enter (out) or normally projected into (in) the hindbrain.

others closer to the midline than in the normal embryos (Fig. 7J) (Yeo et al., 2004). Doubly transgenic embryo that overexpressed both Slit2-GFP and dnPlexinA4-GFP still exhibited similar abnormal axonal pathfinding patterns (Fig. 7K). Therefore, unlike the primary sensory neurons, PlexinA4 appears not to be involved in the Slit2-induced abnormal axonal behavior of the Mauthner neurons, although *plexinA4* is expressed in the Mauthner neurons.

These results indicate that PlexinA4 interacts with the Slit2 signaling specifically to promote axonal branching of Rohon-Beard neurons and the trigeminal sensory ganglion neurons.

Discussion

PlexinA4 is a candidate down stream target gene of Islet2 in the primary sensory neurons of zebrafish embryos

Disappearance of *plexinA4* expression in the primary sensory neurons in the embryos overexpressing LIM^{Isl-2} suggested that PlexinA4 acts as a downstream target gene of Islet2. Injection of AMO against *isl2* mRNA also reduced the expression of *plexinA4* mRNA in these neurons, but could not completely eliminate the expression (data not shown). As AMO against *isl3* had a lethal effect on embryonic development, we could not confirm whether the incomplete effects of AMO against *islt2* mRNA on *plexinA4* expression was due to the redundant roles of Islet3 in these neurons.

In the embryos overexpressing LIM^{Isl-2}, the peripheral axons of the primary sensory neurons were completely eliminated, while their central axons remained intact (Segawa et al., 2001). In contrast to this distinctive effects of functional repression of Islet2, loss of PlexinA4 function induced more limited abnormality, i.e. reduction in axonal branching without severely impairing axonal extension. Some other genes under control of Islet2 may be regulating extension of the peripheral axons of the primary sensory neurons in zebrafish embryos. In fact, expression level of mRNA for other genes such as *pea3* (M. Mieda and H.O., unpublished) and *TrkC1* (H.S. and H.O., unpublished) in the primary sensory neurons are also reduced by overexpression of LIM^{Isl-2}.

PlexinA4 functions in axonal branching

Proteins of the PlexinA family form a co-receptor complex with neuropilin for their ligand, class III secreted semaphorins and function as its signal transducer (Takahashi et al., 1999; Tamagnone et al., 1999; Rohm et al., 2000). Semaphorins are known to induce collapse and repulsion of the growth cone of cultured DRG neurons. In this study, we demonstrated that zebrafish *plexinA4* is a downstream target gene of a LIM-homeodomain-type transcription factor Islet2 and is involved in development of primary sensory neurons especially in branching of the peripheral axons in the primary sensory neurons, rather than mediating the repulsive signaling. In *Drosophila*, PlexA interacts with Sema1a, and promotes branching of the axons of SNa motoneuron (Winberg et al., 1998). This suggests that Plexin function for promotion of axonal branching is evolutionarily conserved. At this point, we have no evidence as to whether the branch-promoting activity of PlexinA4 in the zebrafish primary sensory neurons required semaphorins. In zebrafish, several semaphorin genes were cloned (Halloran et al., 1998; Halloran et al., 1999; Yee et al., 1999; Roos et al., 1999). One of the secreted Sema, *Sema3d*, is expressed in roof plate cells at dorsal midline (Halloran et al., 1999). It would be intriguing to examine its functional relationship to PlexinA4.

Role of PlexinA4 in the Slit signaling

Slit is also known to promote axonal branching of the NGF-responsive neurons in the DRG (Wang et al., 1999). In addition, ubiquitous overexpression of Slit2-GFP in the transgenic zebrafish embryos by heat-shock treatment increased the number of branching of the peripheral axons from the primary sensory neurons such as Rohon-Beard neurons and the trigeminal sensory ganglion neurons (Yeo et al., 2004). Loss of function of PlexinA4 not only reduced the number of branches of the peripheral axons from Rohon-Beard neurons but also counteracted the effects of overexpression of Slit2-GFP on axonal branching. These results suggested that PlexinA4 is necessary for promotion of branching of the peripheral axons of both Rohon-Beard neurons and the trigeminal sensory ganglion neurons by the Slit signaling cascade.

Little is known about why different neurons respond to Slit in different ways. Slit2 is known to be proteolytically processed into a 140 kDa N-terminal fragment and a 55-60 kDa C-terminal fragment in vivo. In addition, the full-length fragment is also found from rat brain extracts. The N-terminal fragment of Slit2 (Slit2-N) but not the full-length Slit2 induces branching (Wang et al., 1999; Nguyen Ba-Charvet et al., 2001). The uncleaved form of Slit2 (Slit2-U) instead acts as an antagonist for Slit2-N in promotion of branching (Nguyen Ba-Charvet et al., 2001).

In contrast to the excessive branching of the peripheral axons of the primary sensory neurons, and defasciculation and deflection of the central axons of the trigeminal sensory ganglion neurons, overexpression of dnPlexinA4-GFP could not rescue the projection errors of Mauthner neurons, which are induced by overexpression of Slit2-GFP (Fig. 7K) (Yeo et al., 2004). Although Mauthner neurons also express both *plexinA4* and *robo3* (Yeo et al., 2004), they start expressing *plexinA4* much later (~21-22 hpf) after their growth cones have already crossed the midline (15-16 hpf). These results indicated that function of PlexinA4 is not involved in collapse or repulsion of the growth cones of the Mauthner neurons at the midline of hindbrain in response to the Slit signals. Therefore, colocalized expression of Robo (Lee et al., 2001; Yeo et al., 2004), a receptor for Slit2, and PlexinA4 may be essential only for promotion of axonal branching in response to Slit but not for the growth cone collapse.

Colocalization of the signaling cascades mediated by Slit and class III semaphorins is also observed in the dendrites of

the pyramidal neurons of the cortex of the mammals. Slit1 acts as a chemorepellent for the axons of these neurons. By contrast, it induces dendritic growth and branching. This dendritic growth and branching is dependent on Slit-Robo system (Whitford et al., 2002). Sema3A act as a chemorepellant for axon and a chemoattractant for apical dendrites. These differences arise from asymmetric cellular localization of soluble guanylate cyclase. Soluble guanylate cyclase is asymmetrically localized to the dendrites, causing higher concentration of cGMP in dendrites than in axons (Polleux et al., 2000). Interaction of Slit and Sema3 signaling may be important for the modeling of the dendrites of cortical pyramidal cells in mammals.

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