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Unc-5/netrin-mediated axonal projection during larval serotonergic nervous system formation in the sea urchin, *Hemicentrotus pulcherrimus*

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ABSTRACT The molecular structure and role of two splice-isoforms of Unc-5 (Hp-Unc-5v1 and v2) in Unc-5/netrin interaction during serotonergic axonal projection were elucidated in this study. Hp-Unc-5v1 was found to be comprised of two immunoglobulin-like domains, two thrombospondin domains in the extracellular region, and ZU-5, DB, and Death domains in the cytoplasmic region, whereas Hp-Unc-5v2 lacked one thrombospondin domain, the transmembrane domain, and all cytoplasmic domains. Hp-Unc-5v1 was transcribed in unfertilized eggs, which continued until the 3-day post-fertilization (-dpf) 2-arm pluteus stage, but was suspended at the mesenchyme blastula stage (mBl), whereas Hp-Unc-5v2 was not transcribed in unfertilized eggs, but was from after fertilization to the same developmental stage of mBl as Hp-Unc-5v1. Relative accumulation of transcripts of both splice-isoforms peaked at the prism stage and declined thereafter, and they were localized at the vegetal pole region of early gastrulae, around the blastopore in mid- to late gastrulae, at fore- and mid-gut regions and on the basal side of dorsal ectoderm in 28-hour post-fertilization prism larvae, and within axons at and after the 2-dpf pluteus stage. Hp-Unc-5v2:GFP was detected in the entire serotonergic cell body and extracellularly on the basal surface of oral ectoderm in 2-dpf plutei and exclusively within axons in 4-dpf plutei. Overexpression of Hp-Unc-5v2 resulted in decreased axonal projection in plutei. Knockdown of *Hp-unc-5v1* and *v2* by morpholino antisense oligonucleotide resulted in severe deficiency of axonal projection. Interference of Unc-5/netrin interaction using an exogenous synthetic IFKSQDFGKWQPY peptide from the VI domain in Hp-netrin, inhibited axonal projection and larval swimming.

KEY WORDS: *axonal projection, Unc-5 variant, netrin, serotonergic axon, sea urchin larvae*

Introduction

The serotonergic nervous system (SNS) of sea urchin larvae plays a major role (Yaguchi and Katow, 2003; Katow *et al*., 2007) along with the dopaminergic regulatory system (Katow *et al*., 2010) and the GABAergic nervous system (Katow *et al*., 2013) in swimming activity. SNS is the last system to emerge among the above three classic nervous systems during the prism larva stage, which is initiated by ganglion formation at the apical ectoderm of the oral lobe (Yaguchi *et al*., 2000; Katow *et al*., 2013). The projection of serotonergic axons occurs under the regulation of netrin on the basal surface of oral ectoderm at the larval apical ganglion during the early pluteus stage. Initial axonal projection occurs toward the netrin-rich dorsal middle-belt that is formed along the antero-posterior axis of larva, and concomitantly during crossing the middle-belt, away from the netrin-rich area in the contralateral direction (Katow 2008).

Such contradictory responses of axons to netrin have been widely acknowledged in commissural axons as they cross the midline in the central nervous system of vertebrates and invertebrates (Van Vactor and Lorenz, 1999; Araújo and Tear, 2003); they are mediated by attraction via Deleted in Colorectal Cancer (DCC) family

Abbreviations used in this paper: -dpf, day post-fertilization; -hpf, hour post-fertilization; SNS, serotonergic nervous system; V1, Hp-Unc-5 variant 1; V2, Hp-Unc-5 variant 2.

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receptors and repulsion via Unc-5 family receptors (Barallobre *et al*., 2005; Sun *et al*., 2011; Norris and Lundquist, 2011).

In this study, we report (1) the identification and structural analysis of two splice-isoforms of Unc-5 receptors of larvae of the sea urchin *Hemicentrotus pulcherrimus*: v1, the canonical molecule, and v2, a short-form molecule, (2) the transcriptional activity of these two variants, (3) their spatiotemporal transcription patterns during development, (4) the potential role of the v2 variant in axonal projection as determined upon their overexpression, (5) loss-of-function study of the v1 variant using morpholino antisense oligonucleotide (MASO), (6) Unc-5/netrin molecular interaction in SNS axonal projections using a synthetic peptide of Hp-netrin, and (7) the effect of Unc-5/netrin interference on larval swimming activity.

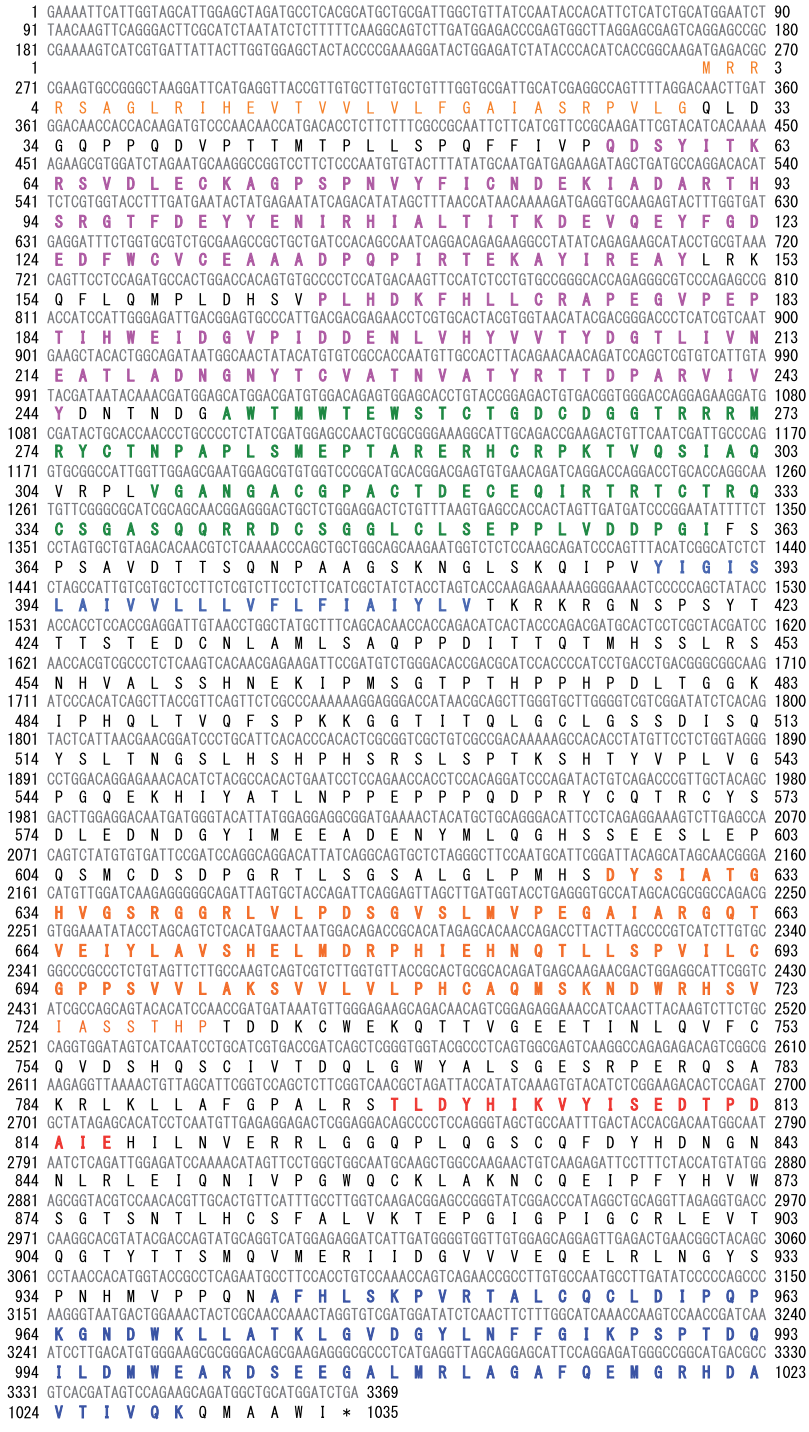

Fig. 1. DNA and protein sequences of Hp-Unc-5v1 inferred from 3,369 bp of cDNA, including a 5′ UTR of 261 bp and an ORF of 3,105 bp, encoding 1,035 amino acids. *Orange letters, signaling peptide region; Pink, immunoglobulin-like domain-1 and -2; Green, thrombospondin type-1 domain-1 and domain-2; Light blue, transmembrane domain; Dark orange, ZU-5 domain; Red, DCC-binding domain; Blue, Death domain.*

Results

Structure of Hp-unc-5

The DNA and protein structure analyses of Unc-5 homolog in this study identified two variants: v1 and v2. The canonical Hp-Unc-5v1 (AB751505) was found to consist of a 5'UTR of 261 bp and an ORF of 3,105 bp that encoded 1,035 amino acids (Fig. 1). On the basis of iPSORT analysis (http://ipsort.hgc.jp/), 30 amino acids at the N-terminus (M^{1}-G^{30}) were found to constitute the signal peptide, which was followed by immunoglobulin-like-1 (Ig-like-1; Q^{57}-Y^{150}) and Ig-like-2 (P^{165}-Y^{244}), thrombospondin-1 (TSP-1; W^{252}-Q^{303}) and TSP-2 domains (V^{308}-I^{361}), a transmembrane domain (Y^{389}-V^{411}; http://www.cbs.dtu.dk/services/TMHMM/), a ZU-5 domain (D^{627}-P^{730}), and a Death domain (A^{943}-K^{1029}) near the C-terminus (Fig. 1). According to Protein Blast analysis, the entire amino acid sequence of Hp-Unc-5v1 was highly similar to Unc5C-like of *S. purpuratus* (XP_003729771; E=0.0) and *Saccoglossus kowalevskii* (NP_001161598; E=0.0), Unc-5 homolog c of *Caenorhabditis elegans* (XP_975281; E=$2e^{-86}$), Unc5C-like of Nile tilapia, *Oreochromis niloticus* (XP_003446151; E=$6e^{-83}$), Unc5B precursor of *Xenopus laevis* (NP_001082302; E=$1e^{-82}$), Unc5C precursor of chicken (NP_989782; E=$1e^{-80}$), Unc5C precursor of mouse *Mus musculus* (NP_03349; E=$1e^{-80}$), and Unc5C of human (AAC67491; E=$2e^{-78}$).

Since a DCC-binding domain (DB domain) that mediates attraction to netrin (Chan *et al*. 1996) was predicted to be located between the ZU-5 and Death domains in mouse Unc-5 (AJ487852; Hong *et al*., 1999), further sequence analysis using ClustalX multiple alignment was applied and identified a region that exhibited a structure highly similar to the DB domain (T^{798}-E^{816}; Fig. 1) in the region between ZU-5 (D^{627}-P^{730}) and Death domains (A^{943}-K^{1029}) of Hp-Unc-5v1. From the presence of the DB domain, the occurrence of Hp-Unc-5v1 interaction with DCC can be predicted, as annotated by SpBase (SPU_025975; http://www.spbase.org/SpBase/search/viewAnnoGeneInfo.php?spu_id=SPU_025975).

According to phylogenetic analysis using full-length amino acid sequences of Unc-5, Unc-5A, B, C, and D and Da homologs of vertebrates and invertebrates, as well as DCC of mice and human as outgroups, Hp-Unc-5v1 was localized in the Unc-5 group of basal deuterostomes, which include sea urchin and the hemichordate *S. kowalevskii* (Supplementary Fig. S1).

In the present study, a shorter sequence of *Hp-unc-*

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   1 GAAAATTCATTGGTAGCATTGGAGCTAGATGCCTCACGCATGCTGCGATTGGCTGTTATCCAATACCACATTCTCATCTGCATGGAATCT  90
  91 TAACAAGTTCAGGGACTTCGCATCTAATATCTCTTTTTCAAGGCAGTCTTGATGGAGACCCGAGTGGCTTAGGAGCGAGTCAGGAGCCGC 180
 181 CGAAAAGTCATCGTGATTATTACTTGGTGGAGCTACTACCCCGAAAGGATACTGGAGATCTATACCCACATCACCGGCAAGATGAGACGC 270
   1                                                                                M  R  R   3
 271 CGAAGTGCCGGGCTAAGGATTCATGAGGTTACCGTTGTGCTTGTGCTGTTTGGTGCGATTGCATCGAGGCCAGTTTTAGGACAACTTGAT 360
   4  R  S  A  G  L  R  I  H  E  V  T  V  V  L  V  L  F  G  A  I  A  S  R  P  V  L  G  Q  L  D  33
 361 GGACAACCACCACAAGATGTCCCAACAACCATGACACCTCTTCTTTCGCCGCAATTCTTCATCGTTCCGCAAGATTCGTACATCACAAAA 450
  34  G  Q  P  P  Q  D  V  P  T  T  M  T  P  L  L  S  P  Q  F  F  I  V  P  Q  D  S  Y  I  T  K  63
 451 AGAAGCGTGGATCTAGAATGCAAGGCCGGTCCTTCTCCCAATGTGTACTTTATATGCAATGATGAGAAGATAGCTGATGCCAGGACACAT 540
  64  R  S  V  D  L  E  C  K  A  G  P  S  P  N  V  Y  F  I  C  N  D  E  K  I  A  D  A  R  T  H  93
 541 TCTCGTGGTACCTTTGATGAATACTATGAGAATATCAGACATATAGCTTTAACCATAACAAAAGATGAGGTGCAAGAGTACTTTGGTGAT 630
  94  S  R  G  T  F  D  E  Y  Y  E  N  I  R  H  I  A  L  T  I  T  K  D  E  V  Q  E  Y  F  G  D  123
 631 GAGGATTTCTGGTGCGTCTGCGAAGCCGCTGCTGATCCACAGCCAATCAGGACAGAGAAGGCCTATATCAGAGAAGCATACCTGCGTAAA 720
 124  E  D  F  W  C  V  C  E  A  A  A  D  P  Q  P  I  R  T  E  K  A  Y  I  R  E  A  Y  L  R  K  153
 721 CAGTTCCTCCAGATGCCACTGGACCACAGTGTGCCCCTCCATGACAAGTTCCATCTCCTGTGCCGGGCACCAGAGGGCGTCCCAGAGCCG 810
 154  Q  F  L  Q  M  P  L  D  H  S  V  P  L  H  D  K  F  H  L  L  C  R  A  P  E  G  V  P  E  P  183
 811 ACCATCCATTGGGAGATTGACGGAGTGCCCATTGACGACGAGAACCTCGTGCACTACGTGGTAACATACGACGGGACCCTCATCGTCAAT 900
 184  T  I  H  W  E  I  D  G  V  P  I  D  D  E  N  L  V  H  Y  V  V  T  Y  D  G  T  L  I  V  N  213
 901 GAAGCTACACTGGCAGATAATGGCAACTATACATGTGTCGCCACCAATGTTGCCACTTACAGAACAACAGATCCAGCTCGTGTCATTGTA 990
 214  E  A  T  L  A  D  N  G  N  Y  T  C  V  A  T  N  V  A  T  Y  R  T  T  D  P  A  R  V  I  V  243
 991 TACGATAATACAAACGATGGAGCATGGACGATGTGGACAGAGTGGAGCACCTGTACCGGAGACTGTGACGGTGGGACCAGGAGAAGGATG 1080
 244  Y  D  N  T  N  D  G  A  W  T  M  W  T  E  W  S  T  C  T  G  D  C  D  G  G  T  R  R  R  M  273
1081 CGATACTGCACCAACCCTGCCCCTCTATCGATGGAGCCAACTGCGCGGGAAAGGCATTGCAGACCGAAGACTGTTCAATCGATTGCCCAG 1170
 274  R  Y  C  T  N  P  A  P  L  S  M  E  P  T  A  R  E  R  H  C  R  P  K  T  V  Q  S  I  A  Q  303
1171 GTGCGGCCATTGGTTGGAGCGAATGGAGCGTGTGGTCCCGCATGCACGGACGAGTGTGAACAGATCAGGACCAGGACCTGCACCAGGCAA 1260
 304  V  R  P  L  V  G  A  N  G  A  C  G  P  A  C  T  D  E  C  E  Q  I  R  T  R  T  C  T  R  Q  333
1261 TGTTCGGGCGCATCGCAGCAACGGAGGGACTGCTCTGGAGGACTCTGTTTAAGTGAGCCACCACGTATGGTGTCTCCCTCTATACTTCTT 1350
 334  C  S  G  A  S  Q  Q  R  R  D  C  S  G  G  L  C  L  S  E  P  P  R  M  V  S  P  S  I  L  L  363
1351 TTGTTTGAAGGCCATGTACAGTAATTGCGAGTATTGCAGCGCTGGGAAAATACTGTTTGTCTATACTCAATATGCTAAAAAATCTATTCT 1440
 364  L  F  E  G  H  V  Q  *                                                                   370
1441 GAAATTCACTAAAAAGGATCATTACTCAAAAAAAAAAAAAAAAAAAA                                           1488
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Fig. 2. DNA and protein sequences of *Hp-unc-5v2* inferred from 1,488 bp of cDNA, including a 5′ UTR of 261 bp, a 3′ UTR of 117 bp, and an ORF of 1,110 bp, encoding 370 amino acids. *Orange letters, signal peptide region; Purple, immunoglobulin-like domain-1 and -2; Green, thrombospondin type-1 domain-1. Underlined sequence, subjected to potential splice-isoform analysis.*

5-like construct (*Hp-unc-5v2*; AB751506) was also cloned, which consisted of a 5′ UTR of 261 bp, a 3′ UTR of 117 bp, and an ORF of 1,110 bp that encoded 370 amino acids (Fig. 2). Its N-terminus end constituted a signaling sequence (M^{1}-G^{30}; http://psort.ims.u-tokyo.ac.jp) that was initiated by four hydrophilic amino acids, three positively charged arginines (R^{2-4}), and a hydrophilic serine (S^{5}), which was identical to that of Hp-Unc-5v1, and then two immunoglobulin-like domains (Ig-1, Q^{57}-Y^{150}; Ig-2, P^{165}-Y^{244}) and one TSP-1 domain (W^{252}-Q^{303}), which were also identical. However, no cytoplasmic domains were detected at the C-terminus region, in which the ORF terminated before the transmembrane domain of *Hp-unc-5v1*. Further homology search using sequences at the 3′ ORF and 3′ UTR regions of *Hp-unc-5v2* and the genome sequence of *Sp-unc-5*, which consisted of 17 exons (Supplementary Fig. S2), detected a highly homologous sequence in an intron region located adjacent to exon 6 of *Sp-unc-5* (XM_003729723; Supplementary Fig. S3). The *Hp-unc-5v2* sequence terminated with poly-A, which suggested that the two *Hp-unc-5* variants may be a post-transcriptionally modified pair of splice-isoforms (Fig. 3; Blobel and Dobberstein, 1975).

Temporal expression pattern of Hp-unc-5

To verify the transcriptional activity of *Hp-unc-5v1* and *-v2* throughout the developmental period, reverse transcription-polymerase chain reaction (RT-PCR) was applied to the mRNA of samples collected at stages from unfertilized eggs to 3-day post-fertilization (-dpf) plutei. *Hp-unc-5v1* was transcribed actively in unfertilized eggs and fertilized eggs, decreased considerably during the mesenchyme blastula stage, and its expression was restored weakly during the early gastrula stage. The transcriptional activity increased considerably from the late gastrula stage and remained high until the 3-dpf pluteus stage (Fig. 4A, *v1*). However, *Hp-unc-5v2* was not transcribed in unfertilized eggs and only weakly in fertilized eggs; then, like the level of *Hp-unc-5v1*, it decreased considerably during the mesenchyme blastula stage. The accumulation of amplicons increased from the early gastrula stage to the prism stage, followed by a significantly higher level at the 3-dpf pluteus stage (Fig. 4A, *v2*). Thus, there was a mid-blastula transition regarding the trends of *Hp-unc-5v1 and v2* transcript levels during the mesenchyme blastula stage. The amplicon accumulation was easily detected by 30-cycle amplification using a set of primers for *Hp-unc-5v1*, whereas those for *Hp-unc-5v2* required two more amplification cycles to be detectable. Although the exact transcription activity ought to have been examined by quantitative PCR, if, for example, the level of transcription of *Hp-unc-5v2* were less than 25% of that of *Hp-unc-5v1*, this difference would be multiplied a billionfold after 30 cycles of amplification, which would provide results suggesting significantly lower transcriptional

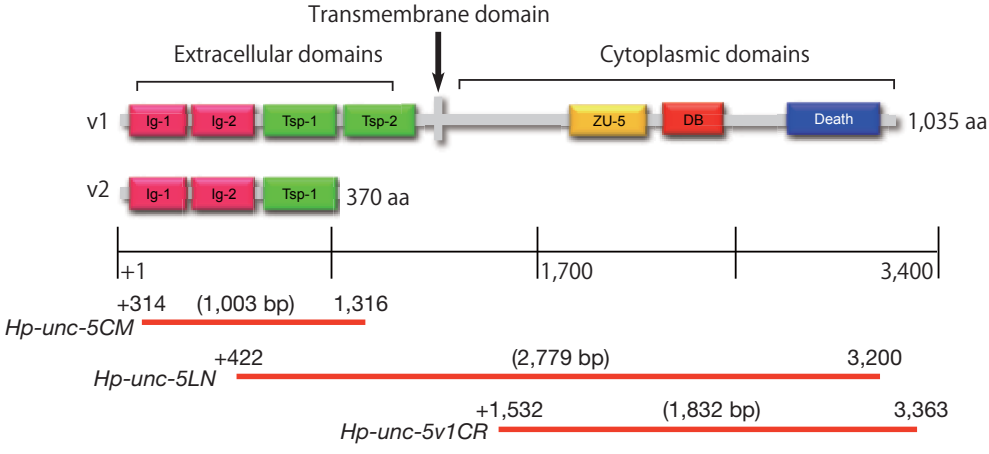

Fig. 3. Domain structure of Hp-Unc-5v1 (v1) and v2 (v2) based on the present amino acid sequence analysis and length and location of probes for whole-mount *in situ* hybridization. *Ig-1, immunoglobulin-like domain-1; Ig-2, immunoglobulin-like domain-2;TSP-1, thrombospondin type-1 domain-1;TSP-2, thrombospondin type-1 domain-2; ZU-5, ZU-5 domain; DB, DCC-binding domain; Death, Death domain. Scale bar: Length that encompassing entire v1 variant. Red bars: location and length of probes.*

activity and thus lower translational activity. This may have meant that the degree to which the intensity of *Hp-unc-5v2* transcription was lower than that of *Hp-unc-5v1*, as presented in Fig. 4A, may be exaggerated, as shown in the following analysis.

Protein expression was examined using antisera raised against Hp-Unc-5v1 and Hp-Unc-5v2 in this study. In immunoblotting, anti-Hp-Unc-5v1 antiserum recognized two bands at regions that corresponded to 83 kDa and 30 kDa (Fig. 4B, lane 1). Thus, translation of Hp-Unc-5v2 was detected. Accordingly, the intensity of the immunoreaction of Hp-Unc-5v1 was significantly stronger than that of Hp-Unc-5v2. The relative molecular mass (*Mr*) of Hp-Unc-5v1 without a signal peptide region was a little smaller than the theoretical value of 108 kDa (PI=5.65) calculated using the ExPASy Compute pl/Mw tool (http://web.expasy.org/compute_pi/), while that of Hp-Unc-5v2 without a signal peptide region was close to the theoretical value of 38 kDa (PI=5.03). Although anti-Hp-Unc-5v2 antiserum recognized only a single band corresponding to 30 kDa (Fig. 4B, lane 2), the intensity of immunoreaction of Hp-Unc-5v2 by anti-Hp-Unc-5v1 antiserum was significantly weaker than that for Hp-Unc-5v1 (Fig. 4C), whereas anti-Hp-Unc-5v2 antiserum indicated higher intensity (Fig. 4D). The expression of both proteins was weaker in unfertilized eggs and fertilized eggs (Figs. 4C, D). However, both Hp-Unc-5v1 (Fig. 4C) and Hp-Unc-5v2 (Fig. 4D) exhibited significantly increased immunoreactions at and after the swimming blastula stage. According to blot image analysis using ImageJ 1.43u open access software (National Institutes of Health, USA; http://rsb.info.nih.gov/ij), the relative intensity of Hp-Unc-5v1 and v2 immunoreaction against tropomyosin indicated low translation activity until the stage of fertilized eggs, which then radically increased at the swimming blastula stage and remained similarly high until the 3-dpf pluteus stage (Fig. 4E). Regarding a few hours difference between the swimming blastula stage and the mesenchyme blastula stage, apparent discrepancy between decreased transcription activity and strong protein expression in mesenchyme blastulae could be due to longer protein life span that was translated during the swimming blastula stage (Fig. 4C).

In situ *hybridization*

The spatiotemporal mRNA transcription pattern of *Hp-unc-5v1* was analyzed by *in situ* hybridization with a probe of 2,779 bp, which encompasses almost the entire ORF of Hp-Unc-5v1 (Fig. 3). Consistent with the results from the RT-PCR analysis as described above, a weak positive signal was detected in ooplasm (Fig. 5A). No accumulation of transcripts was detected from around the 10-hpf morula stage (Fig. 5B) through the 13-hpf swimming blastula stage (Fig. 5C) to the 15-hpf mesenchyme blastula stage (Fig. 5D). At the 18-hpf early gastrula stage, however, weak accumulation of the transcripts was seen at the vegetal plate and the archenteron in the early stage of invagination (Fig. 5E). In 24-hpf late gastrulae, accumulation of the transcript intensified around the blastopore and was lost in the mid- to foregut region (Fig. 5F). At the stage of 28-hpf prism larvae, accumulation of transcripts shifted toward the mid- and foregut region, and, in contrast to that in the previous stage, it was lost from the blastopore region (Fig. 5 G,H). Transcript accumulation was also seen at the dorsal aboral ectoderm region (Fig. 5H, arrow). According to the vegetal plate view, transcript accumulation was not detected in the vegetal plate ectoderm (Fig. 5I). At the 2-dpf 2-arm pluteus stage, while accumulation of the transcripts was retained at the gut region, it was recognized in an additional area, namely, the basal side of the oral lobe ectoderm, with more intensive accumulation at the right and left corners of the oral lobe (Fig. 5J). In 3-dpf 4-arm plutei, transcript accumulation was seen along the circumoral ciliary band region, while that at the digestive organs decreased (Fig. 5K). Sense probes did not detect the transcript accumulation throughout these developmental stages (Fig. 5 a-i). Thus, *Hp-unc-5v1* transcription sites were not limited

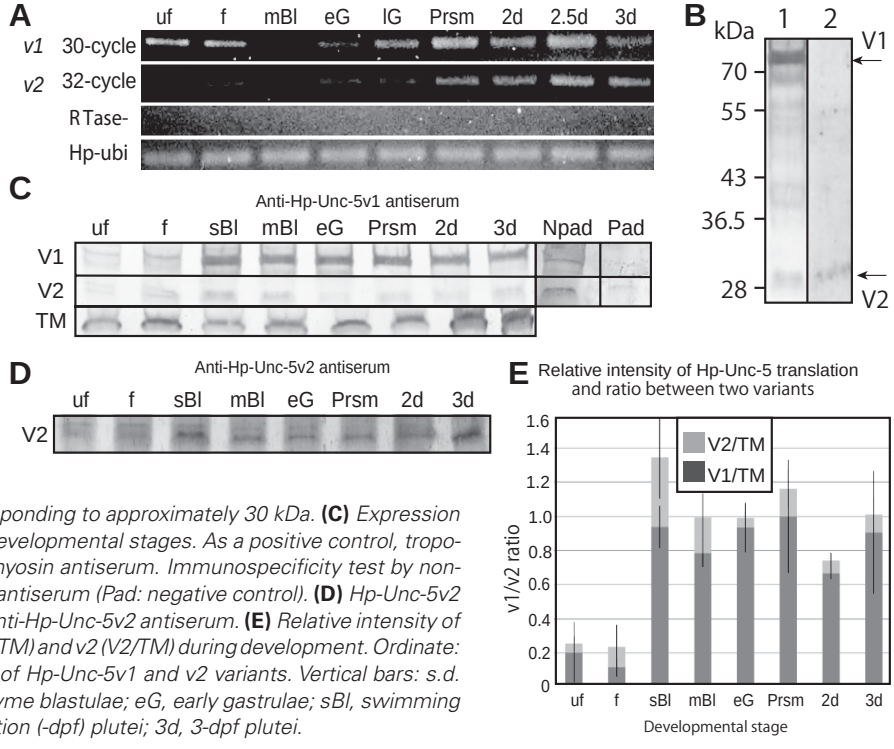

Fig. 4. Transcription and translation patterns of Hp-Unc-5 variants. (A) *Amplicon accumulation pattern by reverse transcription-polymerase chain reaction (RT-PCR) during development.* V1 *30-cycle,* Hp-unc-5v1 *amplicons with 30-cycle RT-PCR;* V2 *32-cycle,* Hp-unc-5v2 *amplicons with 32-cycle RT-PCR; RTase-, without reverse transcriptase;* Hp-ubi, *with* Hp-ubiquitin *primers as a positive control.* **(B)** *Immunoblotting patterns of Hp-Unc-5v1 (lane 1) and Hp-Unc-5v2 (lane 2) using anti-Hp-Unc-5v1 antiserum. V1: V1 variant at region corresponding to approximately 83 kDa. V2: V2 variant at region corresponding to approximately 30 kDa.* **(C)** *Expression pattern of V1 variant (V1) and V2 variant (V2) during developmental stages. As a positive control, tropomyosin (TM) was probed with anti-sea urchin tropomyosin antiserum. Immunospecificity test by non-pre-adsorbed antiserum (Npad) and by pre-adsorbed antiserum (Pad: negative control).* **(D)** *Hp-Unc-5v2 expression pattern during developmental stages by anti-Hp-Unc-5v2 antiserum.* **(E)** *Relative intensity of immunoreaction calculated between Hp-Unc-5v1 (V1/TM) and v2 (V2/TM) during development. Ordinate: Ratio of relative intensity of total Hp-Unc5 and ratio of Hp-Unc-5v1 and v2 variants. Vertical bars: s.d. uf, unfertilized eggs; f, fertilized eggs; mBl, mesenchyme blastulae; eG, early gastrulae; sBl, swimming blastulae; Prsm, prism larvae; 2d, 2-day post-fertilization (-dpf) plutei; 3d, 3-dpf plutei.*

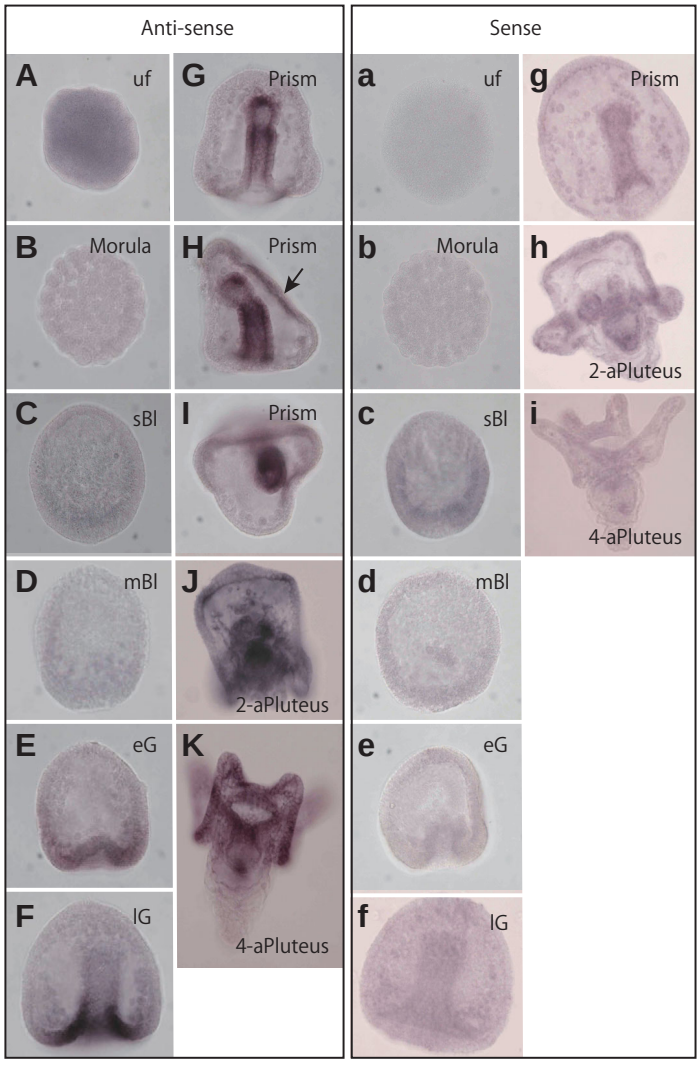

Fig. 5. Whole-mount *in situ* hybridization of *Hp-unc-5v1*. (A,a) *Unfertilized eggs (uf).* **(B,b)** *10-hour post-fertilization (-hpf) morulae.* **(C,c)** *13-hpf swimming blastulae (sBl).* **(D,d)** *15-hpf mesenchyme blastulae (mBl).* **(E,e)** *18-hpf early gastrulae (eG).* **(F,f)** *24-hpf late gastrulae (lG).* **(G,g)** *Oral side view of 28-hpf prism larvae.* **(H)** *Left side view of 28-hpf prism larva.* **(I)** *Vegetal pole view of 28-hpf prism larva.* **(J,h)** *2-day post-fertilization (-dpf) 2-arm pluteus (2-aPluteus).* **(K,i)** *3-dpf 4-arm pluteus (4-aPluteus). Upper case, anti-sense probes; lower case, sense probes.*

to the area where serotonergic axonal projection takes place, but were also seen in the digestive organs, which was suggestive of multiple functions of the protein during development.

The *in situ* hybridization method applied here, using *Hp-unc-5LN* probe that covers both v1 and v2 sequences and *Hp-unc-5v1CR* probe that covers v1-specific region could not specifically identify the location of the v2 variant (data not shown). To compensate for this technical limitation, we deployed overexpression of Green Fluorescent Protein (GFP)-tagged Hp-Unc-5v2 with the expectation that this would enable characterization of the expression sites and potential biological role of Hp-Unc-5v2 exclusively.

Overexpression of Hp-Unc-5v2:GFP

Consistent with the *Hp-unc-5* transcription pattern determined by *in situ* hybridization as described above, Hp-Unc-5v2:GFP was detected in the cytoplasm of serotonergic apical ganglion cells on the basal side (Fig. 6 A-C, yellow arrow) as well as extracellularly on the basal surface of the oral lobe epithelium near the apical ganglion of 3-dpf plutei (Fig. 6 A-C, green arrow). In these plutei, projection of the serotonergic axons was limited to the area immediately beneath the ganglion, while control plutei projected axons away from the apical ganglion (Fig. 6D, arrows). In 4-dpf plutei, Hp-Unc-5v2:GFP was localized in axons, but not extracellularly (Fig. 6 E-G). While axons in control plutei projected far away from the ganglion and branched at this developmental stage (Fig. 6H, arrows), those in plutei overexpressing Hp-Unc-5v2:GFP never projected away from the ganglion, which suggested inhibition of axonal projection by the overexpression of Hp-Unc-5v2 protein. The axonal expression of Hp-Unc-5v2 and its extracellular deposition were also consistently detected by whole-mount immunohistochemistry (Fig. 6I -K). The larvae that were not injected with GFP nor Hp-Unc-5v2:GFP did not show high green background (Fig. 6 L-N), suggesting positive green fluorescence emitted associated with serotonin cells (Fig. 6 A,E,I) is derived from Hp-Unc-5v2:GFP.

This detection of short-type Hp-Unc-5v2 by overexpression of Hp-Unc-5v2:GFP and immunohistochemistry suggested that v2 has a role in axonal projection.

Knockdown of Hp-Unc-5 by MASO

To examine how canonical Hp-Unc-5v1 is involved in the axonal projection process, loss-of-function examination was conducted by knocking down translation of Hp-Unc-5v1 using MASO. In plutei injected with 0.5 mM MASO (data not shown) and those injected with glycerol (Fig. 7A), no visible effect was seen on gangliogenesis, including axonal projection. However, upon the injection of 0.75 mM MASO, the formation of serotonergic axons was severely inhibited (Fig. 7B). This suggests strong involvement of Hp-Unc-5v1 in axonal projection. The embryos that were injected with 1 mM MASO died long before reaching the pluteus stage (data not shown), which indicated a lethal non-specific effect of a high dose of MASO.

Interference of Hp-Unc-5/netrin interaction by an Hp-netrin peptide in serotonergic axonal projection

During axon guidance, Unc-5 interacts with its ligand, Unc-6/Netrin, through an eight-amino-acid sequence, SADFGKTW, in the VI domain of netrin (Lim and Wadsworth, 2002). This eight-amino-acid sequence has been detected in sea urchin netrin (Hp-netrin; Katow 2008) and thus is also implicated in the occurrence of Unc-5/netrin interaction in serotonergic axonal projections in sea urchin plutei. To examine the possible involvement of Hp-Unc-5/netrin interaction through this eight-amino-acid peptide in plutei, a synthetic peptide (I^{177}FKSQDFGKTWQPY^{190}; active peptide) that includes a section that closely resembles this eight-amino-acid sequence was applied to plutei along with a synthetic peptide including the sequence of an eight-amino acid peptide not related to Unc-5/netrin interaction derived from the C-terminus region of Hp-netrin (L^{601}KWRDEWDMRMRKF^{614}; control peptide; Katow 2008). In 2.5-dpf plutei that were incubated with or without the active or control peptides for 24 hours from the prism larva stage, while serotonergic axons projected away from the ganglion in the larvae incubated without peptide (Fig. 8 A,a) and in those with

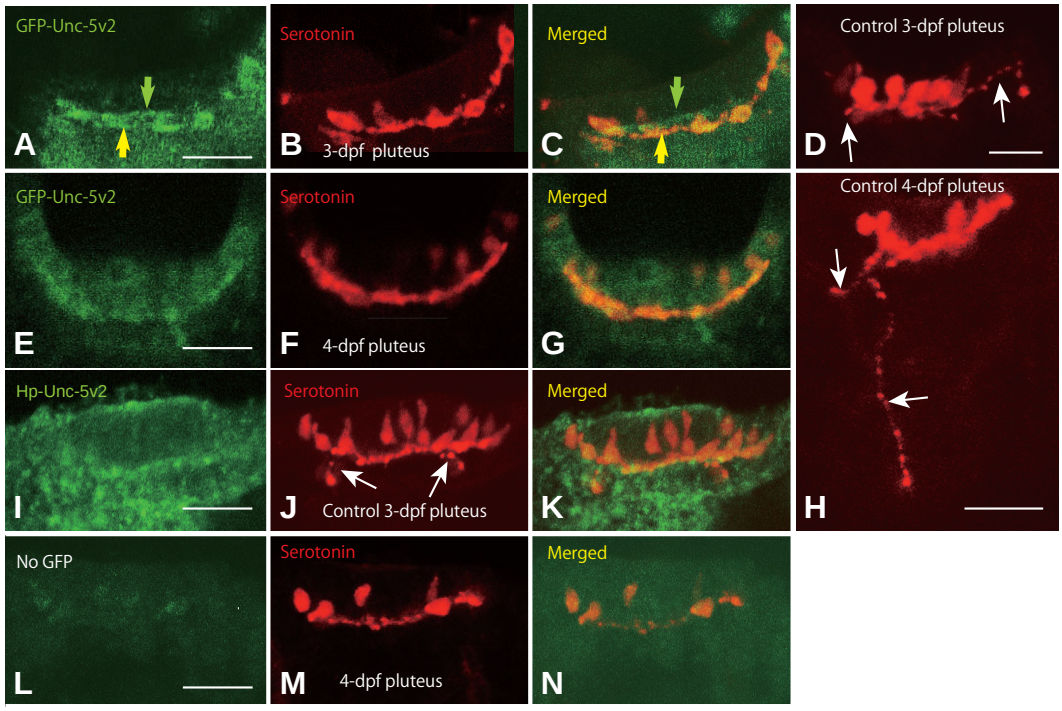

Fig. 6. Whole-mount immunohistochemistry of Hp-Unc-5v2:GFP expression pattern (green) at serotonergic apical ganglion (red). (A-D) *3-day post-fertilization (-dpf) 2-arm pluteus.* **(E-H)** *4-dpf 4-arm pluteus.* **(D), (H)** *Control plutei stained with anti-serotonin antibody.* **(I-K)** *3-dpf pluteus of double-stained whole-mount immunohistochemistry with anti-Hp-Unc-5v2 antiserum (green) and serotonin (red).* **(L)** *4-dpf pluteus that was not injected with GFP nor Hp-Unc-5v2:GFP and probed with 519 nm laser for negative control of green fluorescence emission.* **(M)** *Serotonin stained same larva as (L).* **(N)** *Merged-image between (L,M). Green arrows in (A,C), extracellular Hp-Unc-5v2. Yellow arrows in (A,C), cytoplasmic Hp-Unc-5v2:GFP. Arrows in (D,H,J), axons projected away from the ganglion. Bars: 30 μm.*

7.5 μg/ml control peptide (Fig. 8B), those incubated with 2.5 to 10 μg/ml active peptide showed inhibition of axonal projection in a dose-dependent manner (Fig. 8 C,c-J,j). The active peptide did not affect axonal projection at 2.5μg/ml in the majority of plutei (Fig. 8C, c, K) that was followed by the second major inhibition type of axonal projection with axons in limited area beneath serotonergic cells (type-1 inhibition) and minor group with severe deprivation of axonal projection (type-2 inhibition; Fig. 8K). At 5 μg/ml, normal plutei became rather rare, with those exhibiting type-1 inhibition dominating (Fig. 8 E,e,K), followed by the group with type-2 inhibition (Fig. 8F, f, K). The increase in the size of the type-1 inhibition group compared with that at 2.5 μg/ml was significant. However, at 7.5 μg/ml, while normal plutei remained the smallest group, groups exhibiting type-1 and type-2 inhibition comprised similar proportions of the total (Fig. 8 G,g,H,h,K). At 10 μg/ml, the group with type-2 inhibition was dominant, followed by normally developing plutei. Plutei showing type-1 inhibition became the smallest group in this case (Fig. 8 I,i,J,j,K). The overall response of plutei to the active peptide was dose-dependent in terms of the decrease of axonal projection or even loss of axons.

Inhibitory effect of active peptide on larval swimming behavior

The deficiency of serotonergic axonal projection due to the active peptide may reduce serotonin secretion, which perturbs the formation of the serotonin receptor cell network (SRCN) in the blastocoel (Katow *et al*., 2004) and thus reduces larval swimming activity (Yaguchi and Katow, 2003; Katow *et al*., 2007). Consistent with these previous observations, 3-dpf early 2-arm plutei treated with active peptide did not form a complete blastocoelar SRCN (Fig. 9 A,B) and were associated with a dose-dependent decrease of larval swimming activity, whereas the control peptide did not inhibit larval swimming (Fig. 9C). One-way factorial ANOVA also detected significant deference that was confirmed by Tukey test as post-hoc [Pr (>F) between control and peptide treated and between different concentration of peptide was significant at 0.001348 and 0.007698, respectively].

Discussion

Structures of Hp-Unc-5v1 and v2

Among the several Unc-5 isoforms reported to date, those that lack the transmembrane domain and cytoplasmic domains with an amino acid sequence showing disarrayed at the C-terminus, as in the case of the v2 variant focused on in this study, seem to be rare, but these features have been reported in human Unc-5s (Q6ZN44-1-3, UniProtKB; http://www.uniprot.org/uniprot/?query=unc-5&sort=score).

The present immunoblotting analysis detected two isoforms with *Mr* of 20-25% less than those calculated on the basis of their amino acid sequences. This could be due to unpredicted post-translational modification, such as phosphorylation. According to proteomics analysis using the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/), Hp-Unc-5v1 contains 34 serines and 17 threonines, and Hp-Unc-5v2 contains 5 serines and 8 threonines. Another possibility for this apparent difference in size is that they are highly negatively charged isoforms of less than PI=6 (Shirai *et al*., 2008). The PI values of Hp-Unc-5v1 and Hp-Unc-5v2 were predicted to be 5.65 and 5.03, respectively.

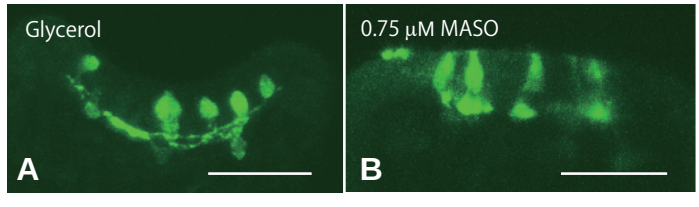

Fig. 7. Inhibition of axonal projection by *Hp-unc-5* morpholino antisense oligonucleotides (MASO) in 2.5-day post-fertilization plutei. (A) *Glycerol-injected larva.* **(B)** *0.75 μM MASO-injected larva. Bars: 30 μm.*

Both Hp-Unc-5v1 and v2 isoforms had an identical 30-amino-acid signal peptide at the N-terminus that was comprised of a positively charged region (n-region; M^1RRRS^5 PI=12.3, Compute pI/Mw; http://web.expasy.org/compute_pi/), a central hydrophobic region (h-region; A^6-A^{24}), and a more polar C-terminal region (c-region; S^{25}-R^{26}) that contains the signal peptidase cleavage site (Von Heijne 1990; Lim and Wadsworth, 2002; Lai *et al*., 2012) and is considered to control protein secretion.

Unc-5 receptors require two extracellular Ig-like domains to interact with netrin (Kruger *et al*., 2004). In the two isoforms of Hp-Unc-5, these domains are conserved, which suggests that they both interact with netrin. Hp-Unc-5v1 is the canonical protein and thus can be anchored to the plasma membrane through its transmembrane domain. However, the absence of this domain and the presence of the signal peptide in Hp-Unc-5v2 suggest that this isoform is secreted into the extracellular space without being anchored to the plasma membrane. This was supported by the observation of Hp-Unc-5v2:GFP in the extracellular space in this study (Fig. 6 A-C). Given its absence of cytoplasmic domains, Hp-Unc-5v2 in the extracellular space may not be able to transmit netrin signals to the cytoplasm, and by pre-emptively binding to the netrin v2 variant could decrease the netrin signal via canonical v1 receptors, as discussed below.

The present transcription and translation analyses of isoforms indicated increased levels in prism larvae, the stage at which axons begin to project at the apical ganglion. Consistent with the RT-PCR analysis in this study, *in situ* hybridization also indicated accumulation of the transcripts at the blastopore region in early gastrulae and in archenteron and endomesoderm during the prism stage. However, these sites of accumulation of transcript were exhibited long before the onset of neurogenesis and are not related to the area where future neurogenesis takes place. This implicates non-neural activity of Hp-Unc-5 in numerous morphogenetic processes, such as cell migration, cell-cell interactions, and cell-extracellular matrix interactions (Sun *et al*. 2011). Neurogenesis-related Hp-Unc-5 transcripts may be those accumulated in serotonergic cell bodies and axons at the apical ganglion from the 2-dpf pluteus stage by *in situ* hybridization and Hp-Unc-5v2:GFP detection.

Isoforms and their role in axonal projection

The role of isoforms of Unc-5 has not been well understood; this includes whether they are limited to only a few species, such as human, mouse, and *C. elegans*, or are widespread (UniProtKB). To date, identification of the presence of two isoforms in the sea urchin is the first of its type for all marine animals.

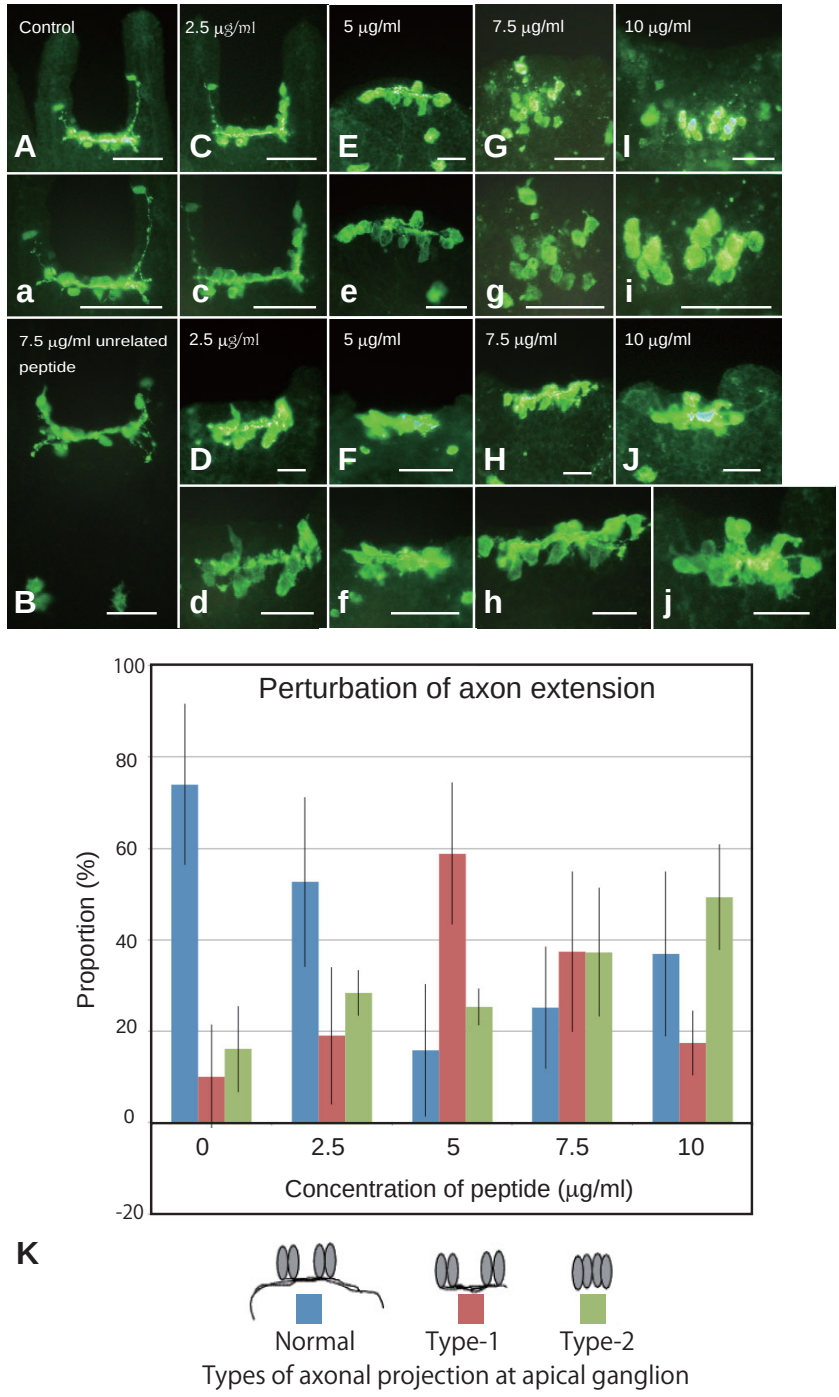

Fig. 8. Interference of Hp-Unc-5/netrin interaction with an SQDFGKTW peptide of Hp-netrin (active peptide) with serotonergic axonal projection in 2.5-day post-fertilization plutei. (A-J, a-j) *whole-mount immunohistochemistry of serotonin.* **(A,a)** *Control normal apical ganglion.* **(B)** *Larvae treated with peptide unrelated to Hp-Unc-5/netrin interaction (LKWRDEWDMRMRKF, control peptide).* **(C,c)** *Predominant type of apical ganglion in larvae treated with 2.5 μm/ml active peptide.* **(D,d)** *Second predominant type of ganglion form of larvae treated with 2.5 μg/ml active peptide.* **(E,e)** *Predominant type of ganglion form of larvae treated with 5 μg/ml active peptide.* **(F,f)** *Second predominant type of ganglion form of larvae treated with 5 μg/ml active peptide.* **(G,g)** *Predominant type of ganglion form of larvae treated with 7.5 μg/ml active peptide.* **(H,h)** *Second predominant type of ganglion form of larvae treated with 7.5 μg/ml active peptide.* **(I,i)** *Predominant type of ganglion form of larvae treated with 10 μg/ml active peptide.* **(J,j)** *Second predominant type of ganglion form of larvae treated with 10 μg/ml active peptide. Upper case, low magnification; lower case, high magnification. Bars: 30 μm.* **(K)** *Proportion of ganglion types with or without active peptide. Vertical bars, s.d.*

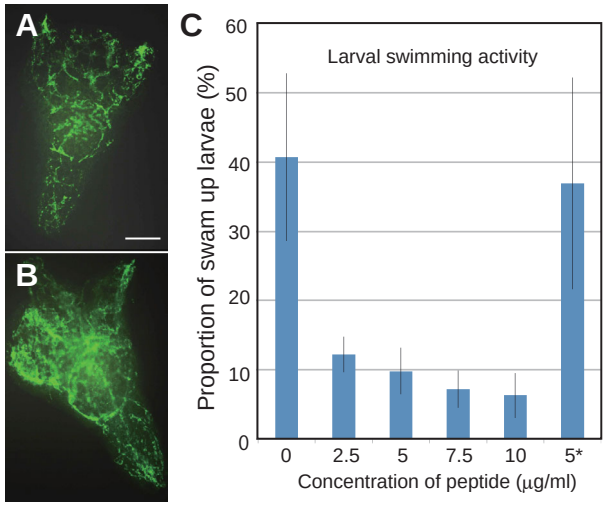

Fig. 9. Swimming activity of 2.5-day post-fertilization larvae in the presence of synthetic SQDFGKTW peptide (active peptide). (A,B) *whole-mount immunohistochemistry of serotonin receptor.* **(A)** *Serotonin receptor network of larvae treated with 5 μg/ml active peptide.* **(B)** *Control untreated larva.* **(C)** *Proportion of actively swimming larvae, which decreased with increasing concentration of peptide. 5* shows the swimming activity of larvae treated with 5 μg/ml LKWRDEWDMRMRKF. Vertical bars, s.d.*

In *C. elegans,* absence of the transmembrane domain abolishes the localization of Unc5 (Ogura and Goshima, 2006), and in the putative null allele *unc-5* (*e53*) that has no cytoplasmic and transmembrane domains, 100% of the ventral class D and dorsal class D motor axons failed to reach the dorsal muscle band (Killeen *et al*., 2002). Thus, lack of a transmembrane domain and cytoplasmic domains in Hp-Unc-5v2 may interfere with the function of Hp-Unc-5v1 to mediate netrin signaling in axonal projections by reducing netrin signaling.

Although no Unc-5 isoforms have yet been identified in *Drosophila*, *Drosophila* homolog of human Down syndrome cell adhesion molecule (DSCAM), which is involved in the formation of axon pathways in the embryonic central nervous system, has multiple isoforms with conserved immunoglobulin (Ig)-like and transmembrane domains, and its alternative splicing can potentially generate more than 38,000 isoforms. Different isoforms exhibit different binding specificity. These isoforms seem to contribute to the specificity of neuronal connectivity (Schmucker *et al*., 2000; Wojtowicz *et al*., 2004).

In the present study, extracellular Hp-Unc-5v2:GFP was detected by the 3-dpf pluteus stage and disappeared from extracellular space in one day, which indicated its short-lived extracellular presence. These Hp-Unc-5v2-overexpressing plutei did not project axons away from the ganglion and thus showed similar morphological deficiencies to plutei that had been treated with a synthetic peptide derived from netrin VI domain (Fig. 8). The netrin VI domain binds to Ig-like domains of Unc-5 through the sequence contained within this synthetic peptide (Lim and Wadsworth, 2002; Geisbrecht *et al*., 2003). Since multiple binding sites are required to send netrin signal to Unc-5 and to the cytoplasm, the inhibition of one of these binding sites using the synthetic peptide (a pseudo-netrin) may inhibit or alter signal transmission by canonical netrin to Unc-5, such as through tyrosine phosphorylation (Kruger *et al*., 2004). Apparently, the pseudo-netrin peptide inhibited initial serotonergic axonal projection by lowering relative concentration of netrin, which suggests the involvement of Unc-5/netrin interaction during serotonergic axonal projection. The above morphological similarities between the effect of Hp-Unc-5v2 overexpression and that upon application of a pseudo-netrin peptide on serotonergic axonal projection could suggest that Hp-Unc-5v2 modifies Unc-5/netrin interaction by a molecular mechanism similar to that of pseudo-netrin peptide. Particularly regarding the repulsion of netrin by Unc-5 of axons (Barallobre *et al*., 2005; Norris and Lundquist, 2011), some factor(s) that changes the repulsive response to netrin to an attractive one must be involved in contralateral serotonergic axonal projection at the same time as the axons cross the netrin-rich dorsal middle-belt in plutei.

Taking together the previous studies described above and the present results from Hp-Unc-5v2:GFP overexpression and pseudo-netrin peptide application, the contradictory responses of serotonergic axons to netrin may be dependent on the combination of Hp-Unc-5v1 and v2 isoforms. Apparent attraction of axons to the netrin-rich middle-belt of the larval axis may be due to the presence of the Hp-Unc-5v2 isoform during the early period of axonal projection, as was seen in 3-dpf plutei. The isoform may reduce the repulsive response of Unc-5 to netrin at the apical ganglion during the early period of axonal projection and subsequently gradually increase this repulsive response to netrin during crossing the middle-belt leaving a netrin-rich area of the apical ganglion from which axons can be projected further to contralateral areas of larva where the netrin concentration is lower and the targets of axons reside (Katow 2008).

However, the actions of this combination of isoforms may not necessarily be the only axon guidance mechanism in sea urchin larvae. DCC of sea urchin could also coordinate with Unc-5 or play a distinctive role in altering the axon response to netrin from repulsion to attraction, or *vice versa* (Hong *et al*., 1999). However, while the presence of DCC has been predicted by SpBase (SPU_025975; Katow 2008), no further detail on the expression and role is available at present.

Unc-5 expression sites in neuron

The response of axons to their projection cues is considered to be localized at growth cones, which are cytoskeletal structures (Tanaka and Sabry, 1995; Norris and Lundquist, 2011), and to be carried out through tyrosine phosphorylation of cytoplasmic domains of Unc-5 (Killeen *et al*., 2002; Kruger *et al*., 2004; Barallobre *et al*., 2005). However, by *in situ* hybridization of Hp-Unc-5v1 in 2-arm plutei, overexpression of Hp-Unc-5v2:GFP, and immunohistochemistry of Hp-Unc-5v2 in 3-dpf and 4-dpf plutei in the present study, these proteins were found to be localized throughout the serotonergic cell body or, in later developmental stages, throughout the axonal region. These observations are not only consistent with previous immunohistochemical observations of UNC5H1 (Williams *et al*., 2003) and UNC-5:GFP in *C. elegans* (Killeen *et al*., 2002), but also suggest that axons receive netrin information at the growth cone, as well as throughout the axonal region.

Materials and Methods

Gamete preparation

Gametes of the sea urchin *H. pulcherrimus* were collected and inseminated, and the zygotes were incubated at 18°C as described previously

TABLE 1

PRIMER SETS FOR CLONING OF *HP-UNC-5*

Primer name: Abbreviation	Sequence
Unc-5 forward primer: T1-1	5'-TCGTTCCGCAAGATTCGTACA-3'
Unc-5 reverse primer: T1-2	5'-GCTTCTCTGATATAGGCCTTC-3'
ZD forward primer: T1-3	5'-ACGAGTGTGAACAGATCAGG-3'
ZD reverse primer: T1-4	5'-TCTGTCCATTAGTTCATGTG-3'
ZD sequence primer: T1-5	5'-GCACAACCACCAGACATCAC-3'
DB forward primer: T1-6	5'-TGCTACCAGATTCAGGAGTT-3'
DB reverse primer: T1-7	5'-ATCCATCGACACCTAGTTTG-3'
DB sequence primer: T1-8	5'-CAGGTGGATAGTCATCAATC-3'
Exon13 forward primer: T1-9	5'-CCTTTCTACCATGTATGGAGC-3'
Exon16 reverse primer: T1-10	5'-ATTGGCACAAGGCGGTTCTGA-3'
DD reverse primer: T1-11	5'-TCAGATCCATGCAGCCATCT-3'
V2 forward primer: T1-12	5'-ACAGAGTGGAGCACCTGTA-3'
V2 specific reverse primer: T1-13	5'-AGCGCTGCAATACTCGCAA-3'

(Katow *et al*., 2010) until the developmental stages described in the text.

Total RNA extraction and cDNA preparation

Total RNA was isolated from unfertilized eggs, eggs 30 minutes after fertilization (fertilized eggs), 15-hpf mesenchyme blastulae, 18-hpf early gastrulae, 24-hpf late gastrulae, 28-hpf prism larvae, 2-dpf 2-arm plutei, 2.5-dpf 2-arm plutei, and 3-dpf 4-arm plutei by solubilization in ISOGEN (NIPPON GENE, Tokyo, Japan). cDNA was prepared by reverse transcription using Oligo d(T) primer (Novagen, Madison, WI, USA) and Superscript II (Invitrogen, Tokyo, Japan).

RT-PCR and DNA sequencing

Primers used for cloning of Unc-5 are summarized in Table 1. RT-PCR was conducted using T1-1 and -2 primers that were designed on the basis of the predicted Unc-5 sequence of *Strongylocentrotus purpuratus* (NCBI, XM_786929), which is known to be a sister species of *H. pulcherrimus* used in this study (Katow *et al*., 2013), and Ex Taq DNA polymerase (TAKARA, Otsu, Japan).

The amplicons from the RT-PCR were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and ligated to pGEM-T Easy vector (Promega). The vector was introduced into JM109 cells (Promega), transformed, and incubated in Super Optimal broth with Catabolite repression (SOC) medium (Invitrogen) for 2 hours at 37°C. Then, 100 μl of the JM109 cell suspension was plated on Luria Broth (LB)/ampicillin/Isopropyl β-D-1-thiogalactopyranoside (IPTG)/X-Gal plates, and incubated overnight at 37°C. The presence of colonies of transformed cells was examined by colony direct PCR. Positive colonies were further incubated in LB medium for 16 hours at 37°C. Plasmids were collected using GeneElute Plasmid Mini-Prep Kit (Sigma, St. Louis, USA), cycle-sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan), and analyzed with an ABI PRISM310 DNA Genetic Analyzer (PE Applied Biosystems, Tokyo, Japan).

The ZU-5 domain of Unc-5 was sequenced as described above for cloning using T1-3 and -4 primers designed by referring to the sequences that were obtained from 3' Rapid amplification of cDNA ends (RACE) in the present study and the predicted gene sequence of Sp-Unc-5 (SPU_010776; SpBase http://www.spbase.org/SpBase/search). Supplemental sequencing was conducted using T1-5 primer. The region downstream of Unc-5 was sequenced using T1-6 and -7 primers. Supplemental sequencing was conducted using T1-8 primer. Sequences from exon 13 to 16 were predicted in SPU_010776 and sequenced using T1-9 and -10 primers (Table 1). This set of primers was also used for RT-PCR of *Hp-unc-5v1*. Death domain was sequenced using T1-9 and -11 primers.

The expression pattern of *Hp-unc-5v2* throughout the different developmental stages was analyzed by RT-PCR using T1-12 primer for the upstream sequence of *Hp-unc-5* and T1-13 primer for the UTR sequence immediately after the stop codon of *Hp-unc-5v2*.

Rapid amplification of cDNA ends

The primers used for RACE are summarized in Table 2. The Oligo probe of GeneRacer Kit (Invitrogen) was exposed to the total RNA extracted from 2.5-dpf plutei, and 5'-RACE template was prepared by RT-PCR using Oligo d(T) primer of GeneRacer Kit (Invitrogen). For the isolation of 5' UTR, the following PCR was conducted using T2-1 and -2 primers that were designed on the basis of the sequence of *Hp-unc-5* that was cloned in this study. Nested PCR was conducted using T2-3 and -4 primers with the above amplicons as a template, and sequenced as described above.

An aliquot of the above cDNA prepared from the total RNA of 2.5-dpf plutei was used as a template for 3' RACE PCR with T2-5 and -6 primers, and the subsequent nested PCR was conducted using T2-5 and -7 primers. Supplemental sequencing was conducted using T2-8 primer. The amplicons were dissected from agarose gels, purified using Wizard SV Gel and PCR Clean-Up System (Promega), and sequenced as stated above.

Whole-mount in situ hybridization

Plasmids that contained (1) a 2,779-bp sequence from the 5' end (+422) to the 3' end (+3,200), (2) a 1,832-bp sequence from the 5' end (+1532) to the 3' end (+3,363), and (3) a 1,003-bp sequence from the 5' end (+314) to the 3' end (+1,316) were prepared (Fig. 3). The Dig-tagged probes were transcribed for three hours using SP6/T7 RNA polymerase (Roche, Mannheim, Germany). The probes were named as follows: *Hp-unc-5* long probe (*Hp-unc-5LN*), *Hp-unc-5v1* probe for the cytoplasmic region (*Hp-unc-5v1CR*), and probe for the common region between *Hp-unc-5v1* and *Hp-unc-5v2* (*Hp-unc-5CM*).

Unfertilized eggs, fertilized eggs, and 10-hpf morulae were fixed overnight at 4°C with a mixture of 4% paraformaldehyde in 32.5% FSW, 32.5 mM 3-(N-morpholino)propanesulfonic acid (MOPS; pH 7.0), and 0.5 M NaCl. Then, 13-hpf swimming and 15-hpf mesenchyme blastulae, 18-hpf early and 24-hpf late gastrulae, 28-hpf prism larvae, and 2-dpf 2-arm and 3-dpf 4-arm plutei were fixed with a mixture of 4% paraformaldehyde, 0.1 M MOPS (pH 7.0), and 0.5 M NaCl.

Before the onset of hybridization, the above samples were treated with 2 μg/ml Proteinase K in MOPS buffer for 15 minutes at 37°C, washed twice with MOPS buffer, fixed with 4% paraformaldehyde in MOPS buffer for 20 minutes, washed with MOPS buffer three times, and incubated with hybridization buffer [70% formaldehyde, 0.1 M MOPS (pH7.0), 0.5 M NaCl, 0.1% Tween-20, 1 mg/ml bovine serum albumin (BSA)] for 3 hours at 51°C for *Hp-unc-5LN* and *Hp-unc-5v1CR* probes, and at 48°C for *Hp-unc-5CM* probe, and hybridized with probes at 0.5 ng/μl for seven days at 51°C for

TABLE 2

PRIMER SETS FOR RACE

Primer name: Abbreviation	Sequence
5' GeneRacer primer: T2-1	5'-CGACTGGAGCACGAGGACACTGA-3'
5' RACE reverse primer: T2-2	5'-TCTCTGTCCTGATTGGCTGTGGAT-3'
Unc-5 nested primer: T2-3	5'-GAGAATGTGTCCTGGCATCAGCTA-3'
5' GeneRacer nested primer: T2-4	5'-GGACACTGACATGGACTGAAGGAGTA-3'
3' GeneRacer primer: T2-5	5'-CGCTACGTAACGGCATGACAGTG-3'
3' RACE forward primer: T2-6	5'-GCCGGTCCTTCTCCCAATGTGTACT-3'
3' RACE nested primer: T2-7	5'-GCCAATCAGGACAGAGAAGGCCTATA-3'
3' RACE sequence primer: T2-8	5'-CAGCTCGTGTCATTGTATACGAT-3'

Hp-unc-5LN and *Hp-unc-5v1CR* probes and at 48°C for *Hp-unc-5CM* probe. The post-hybridization was followed by washing with hybridization buffer for six hours at 57°C for *Hp-unc-5LN*, 54°C for *Hp-unc-5v1CR*, and 51°C for *Hp-unc-5CM* probes. After further washing with MOPS buffer five times, the blocking was conducted twice with MOPS buffer containing 10 mg/ml BSA for 15 minutes at ambient temperature and MOPS buffer containing 10% goat serum and 1 mg/ml BSA for 15 minutes at 37°C. Alkaline phosphatase (AP)-tagged sheep anti-Dig antibody (Roche) was diluted in MOPS buffer containing 1% goat serum and 0.1 mg/ml BSA, and incubated overnight at ambient temperature. Then, the samples were washed five times with MOPS buffer, replaced with fresh buffer for 30 minutes, and developed in nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate dissolved in AP buffer (pH 9.0) containing 10% dimethyl formamide overnight at ambient temperature. The samples were further washed briefly with MOPS buffer twice, and replaced with 50% glycerol for examination under a light field microscope.

Generation of GFP-tagged Hp-Unc-5v2

GFP-inserted pCS2+ vector (pCS2+GFP) was generated by ligating the coding sequence of tagGFP2-C GFP vector (Evrogen, Moscow, Russia) to pCS2+ vector. The coding sequence of *Hp-unc-5v2* was amplified by the following primer set from cDNA template of 36-hpf prism larvae.

F-v2: 5'-TCACCGAATTCATGAGACGCCGAAGTGCCGG-3'

R-v2: 5'-ACTCGCTCGAGCTGTACATGGCCTTCAAACA-3'

The amplicons were cleaved with EcoRI and XhoI and ligated to pCS2+GFP. The vector was cleaved with NcoI, and capped mRNA was synthesized using mMESSAGE mMACHINE SP6 Kit (Ambion, Austin, TX, USA). Then, 3 μg/μl of the synthetic mRNA was microinjected into each unfertilized eggs. They were inseminated and raised in a culture dish in an incubator at 18°C until the stage described in the text.

Hp-unc-5 knockdown by MASO

Hp-unc-5 MASO (5'-CGGCACTTCGGCGTCTCATCTTG-3') was diluted with glycerol at 0.5 mM, 0.75 mM, and 1 mM (final concentrations in the eggs were about 10 μM, 12 μM, and 15 μM, respectively), injected into unfertilized eggs. They were inseminated and incubated for 60 hours at 18°C. Negative control was injected with glycerol into unfertilized eggs and incubated as described above.

At the 2.5-dpf pluteus stage, the larvae were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline [PBST; 40 mM NaH_2PO_4-$2H_2O$, 60 mM $NaHPO_4$-$12H_2O$, 10 mM NaCl, 0.3 mM KCl, 0.05% Tween-20 (pH 7.0)] for 15 minutes at ambient temperature, washed three times in PBST (10 minutes each), and used for whole-mount immunohistochemistry (WMIHC) as described below.

Generation of antisera

A synthetic 24-amino-acid peptide, A^{251}WTMWTEWSTCTGDCDG-GTRRRMR^{274}, which contained a sequence present in the TSP-1 domain of both v1 and v2 variants, was chosen on the basis of physico-chemical property analysis, namely, Pôle BioInformatique Lyonnais Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_pcprof.html). For v2 variant-specific antiserum, a v2 variant-specific 13-amino-acid peptide, S^{358}PSILLLFEGHVQ^{370}, from the carboxyl terminus was used (Supplementary Fig. S4). These synthetic peptides were tagged with keyhole limpet hemocyanin through the addition of cysteine to A^{251} for anti-Hp-Unc-5 antiserum and to S^{358} for anti-Hp-Unc-5v2 antiserum, diluted in Gerbu adjuvant 100 (Gerbu Biotechnik GmbH, Gailberg, Germany; diluted 1:2 in sterilized distilled water), and inoculated as previously described (Katow *et al*., 2004).

Immunospecificity of antisera was tested by immunoblotting using 1 mg/ml lyophilized mesenchyme blastulae, as described previously (Katow 2008). Anti-sera were diluted in Tris buffer [TBST; 50 mM Tris-HCl (pH 7.0), 0.17 M NaCl, and 0.05% Tween 20] at 1:3000 for anti-Hp-Unc-5v1 antiserum and at 1:100 for anti-Hp-Unc-5v2 antiserum and incubated with 10 mg/ml antigen peptides for two hours at ambient temperature (pre-adsorbed antisera). Immunoblotting was conducted as described below and the blots were pre-incubated with 5% skimmed milk for one hour; 200 μl of pre-adsorbed antisera or non-pre-adsorbed antisera were applied to 1 x 1 cm pieces of the blots that contained corresponding area of immunopositive band detected by non-pre-adsorbed antisera and were placed in small Parafilm boats on a rocking deck for two hours at ambient temperature (Katow *et al*., 2013).

Immunoblotting

Unfertilized eggs, fertilized eggs, swimming and mesenchyme blastulae, early gastrulae, prism larvae, and 2-dpf and 3-dpf plutei were lyophilized, dissolved at 1 mg/ml in sodium-dodecyl sulfate acrylamide gel electrophoresis (SDS-PAGE) sample buffer with 2-mercaptoethanol, blotted to nitrocellulose filters, and incubated with antisera raised in the present study as described previously (Katow *et al*., 2004). The relative intensity of immunoreaction was examined using tropomyosin as a standard (Katow *et al.,* 2013), and scanned images of the immunoblotting were analyzed using ImageJ 1.43u (NIH). The analysis was repeated three times using three different immunoblots conducted using three different sets of samples.

Whole-mount immunohistochemistry

Hp-unc-5 MASO-injected 2.5-dpf plutei and synthetic peptide-treated 3-dpf plutei were fixed with 4% paraformaldehyde and incubated with rabbit anti-serotonin-antibody (1:500 diluted in PBST; Sigma) or mouse anti-serotonin-receptor antiserum (1:500 diluted in PBST; Katow *et al.,* 2004). These primary antibodies were visualized using Alexa Fluor 488- or 594-tagged sheep anti-mouse IgG or anti-rabbit IgG antibodies (Invitrogen; diluted 1:500 in PBST), and examined under a Micro Radiance 2000 Confocal Laser Scanning Microscope (BioRad, Hercules, CA, USA) as described previously (Katow *et al*., 2013). The resulting images were analyzed using an ImageJ 1.43u (NIH) and Photoshop CS5 Extended (Adobe Systems Inc., San Jose, CA, USA).

Inhibition of Unc-5 interaction by synthetic peptide during serotonergic axonal projection and of larval swimming

To examine possible interaction between Hp-netrin and Hp-Unc-5, a synthetic peptide (I^{178}FKSQDFGKTWQPY^{191}; active peptide) containing a sequence located at the VI domain near the amino terminus of Hp-netrin (Katow 2008) was applied to the larvae obtained from three different females in 24-well plates for 28 hours at 18°C from the 34-hpf prism stage to the 2.5-dpf pluteus stage. This was done in the presence of 2.5 μg/ml, 5 μg/ml, 7.5 μg/ml, or 10 μg/ml synthetic peptide and the L^{600}KWRDEWDMRMRKF^{614} peptide, which contains a sequence located at the carboxyl terminus of Hp-netrin that is not related to Unc-5/netrin interaction (inactive peptide; Katow 2008). For swimming assay, triplicate wells of 24-well plates were filled with 3 ml of larvae, and those that swam up near the water surface at the time described above were counted under a dissection microscope. The experiment was repeated three times using a total of 545 larvae for 0 μg/ml, 652 for 2.5 μg/ml, 534 for 5 μg/ml, 954 for 7.5 μg/ml, and 739 for 10 μg/ml peptide.

Immediately after counting their number, the larvae were processed for WMIHC as described above. Statistical analysis was conducted by ANOVA that was calculated using R ver 2.13.2. (http://aoki2.si.gunma-u.ac.jp/R/?src?tukey.R).

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