Dcc Regulates Asymmetric Outgrowth of Forebrain Neurons in Zebrafish

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Abstract

The guidance receptor DCC (deleted in colorectal cancer) ortholog UNC-40 regulates neuronal asymmetry development in Caenorhabditis elegans, but it is not known whether DCC plays a role in the specification of neuronal polarity in vertebrates. To examine the roles of DCC in neuronal asymmetry regulation in vertebrates, we studied zebrafish anterior dorsal telencephalon (ADt) neuronal axons. We generated transgenic zebrafish animals expressing the photo-convertible fluorescent protein Kaede in ADt neurons and then photo-converted Kaede to label specifically the ADt neuron axons. We found that ADt axons normally project ventrally. Knock down of Dcc function by injecting antisense morpholino oligonucleotides caused the ADt neurons to project axons dorsally. To examine the axon projection pattern of individual ADt neurons, we labeled single ADt neurons using a forebrain-specific promoter to drive fluorescent protein expression. We found that individual ADt neurons projected axons dorsally or formed multiple processes after morpholino knock down of Dcc function. We further found that knock down of the Dcc ligand, Netrin1, also caused ADt neurons to project axons dorsally. Knockdown of Neogenin1, a guidance receptor closely related to Dcc, enhanced the formation of aberrant dorsal axons in embryos injected with Dcc morpholino. These experiments provide the first evidence that Dcc regulates polarized axon initiation and asymmetric outgrowth of forebrain neurons in vertebrates.

Introduction

Neurons are polarized cells. The acquisition of correct polarity is a critical step in neuronal differentiation and subsequent circuit formation. Intracellular kinases and polarity proteins regulate cytoskeletal dynamics and intrinsic cellular programs that determine neuronal polarity [1–8]. In vivo, developing neurons encounter extracellular cues that promote asymmetric growth and direct the formation of correct polarity [9–14]. In Caenorhabditis elegans, the guidance receptor UNC-40 (DCC, deleted in colorectal cancer) is localized to the ventral side of the hermaphrodite specific neurons (HSN) before they extend axons ventrally towards the ventral nerve cord, where the UNC-6 (netrin) guidance cue is secreted [11]. Mutations in UNC-40 may result in randomly oriented neuronal processes. In unc-40 loss of function mutants or in unc-40 missense plus unc-6 double mutants, HSN axons extend anteriorly, posteriorly, or dorsally, instead of towards the correct ventral position [13]. These studies suggest that the guidance receptor DCC is a key regulator of neuronal asymmetry in C. elegans. The functions of DCC in axon guidance and cell migration are conserved in bilateria [16–19], but few studies have examined the roles of DCC in neuronal polarity regulation in vertebrates.

We studied Dcc function in the zebrafish forebrain and found that it regulates asymmetric neuronal growth. Utilizing transgenic animals expressing fluorescent proteins, we determined that anterior dorsal telencephalic (ADt) neurons project axons ventrally. ADt neurons express Dcc before the axons emerge, and the Dcc ligand, Netrin1, is expressed in ventrally located cells. We found that knock down of Dcc function by antisense morpholino oligonucleotides causes ADt neurons to project aberrant axons dorsally. We further examined the axon projection patterns of individual ADt neurons and found that ADt neurons project axons dorsally or form multiple processes after knockdown of Dcc function. In addition, we found that knock down of the Dcc ligand, Netrin1, also caused the ADt neurons to project axons dorsally. Knockdown of Neogenin1, a guidance receptor closely related to Dcc, enhanced the formation of aberrant dorsal axons in embryos injected with Dcc morpholino. These data show that Dcc regulates polarized axon initiation and asymmetric outgrowth of neurons in a vertebrate forebrain.

Results

ADt Neurons Project Axons Ventrally

In the developing zebrafish forebrain, a cluster of cells are specified as neurons in the dorsal rostral region of the neural tube. By the pharyngula period, these telencephalic neurons have extended axons along a commissural tract (the anterior commissure, AC) and a descending tract (the supraoptic tract, SOT) [20]. In the anterior dorsal telencephalon, ADt neurons express lhx5 (Fig. 1A), which encodes a LIM homeodomain protein that
regulates the antagonism of Wnt signaling, thus promoting forebrain development [21]. To visualize the lhx5 expressing ADt neurons and their axons in live intact animals, we generated transgenic zebrafish expressing the photo-convertible fluorescent protein Kaede [22] using a bacterial artificial chromosome (BAC) homologous recombination method [23,24]. The BAC clone carried approximately 200 kb of zebrafish genomic sequence, of which approximately 110 kb was upstream of the transcripational start site of the lhx5 gene (Fig. 1B). The modified BAC construct contained Kaede coding sequence that replaced the first exon of the lhx5 gene. Two lines of Tg(lhx5BAC:Kaede) stable transgenic fish were obtained after screening 80 of the modified BAC injected fish. The expression patterns of the Kaede protein were identical between the two lines. We maintained one of the Tg(lhx5BAC:Kaede) lines (referred to as lhx5:Kaede hereafter).

By the mid-pharyngula period at 36 hpf, fluorescent signal from transgenic Kaede expression was observed in the somata of the ADt neurons and axon tracts in the forebrain (Fig. 1C). To ascertain which axon tracts originated from the ADt neurons, we photo-converted Kaede protein in the ADt neuronal somata from the right side of the telencephalon (Fig. 1C). Projecting axons from the ADt neurons were distributed among the dividing cells, and mosaic expression of the specific phenotypic score matrix we employed. Based on this analysis, the dcc-MO injected embryos (a.p.s = 0.983) were significantly different from the standard control morpholino injected animals (a.p.s = 0.063) (Fig. 3C, p = 7.01 × 10⁻³). Similar statistical results were obtained using the original quantification data and the ANOVA method (Fig. S1). Injection with a lower dosage of dcc-MO (3 ng per embryo) reduced the severity (a.p.s = 0.500) of the aberrant dorsal axons. Injection with a higher dosage of dcc-MO (12 ng per embryo) caused early death in about 30% of the injected embryos (52 out of 156). The intensity of the abnormal dorsal axon was increased in the remaining embryos, but the differences were not statistically significant (a.p.s = 1.13, p = 0.425 versus embryos injected with 6 ng of dcc-MO). Embryos were injected with 6 ng of dcc-MO in following experiments.

We used two methods to validate the specificity of the dcc-MO knockdowns. We examined phenotypes caused by injection of a mismatch control morpholino, and we tested for rescue of the dcc-MO knockdown phenotypes by coinjection of a dcc-MO resistant mRNA (dcc-R-mRNA, Fig. 3C). We designed a mismatch control morpholino that contained 5 mismatch bases. In embryos injected with the mismatch control morpholino at the same dose as the dcc-MO, we rarely observed dorsally projecting processes (a.p.s = 0.065, p = 1.31 × 10⁻⁶ versus dcc-MO injected group). To construct the dcc mRNA whose translation was resistant to the blocking effects of the dcc-MO, we introduced 7 silent mutations into the dcc mRNA sequence. After co-injection of the dcc-MO and the dcc mRNA with silent mutations, the occurrence and severity of the dorsal processes were significantly reduced (a.p.s = 0.281, p = 4.62 × 10⁻⁴ versus dcc-MO injected embryos). Similar statistical results were obtained using the original quantification data and the ANOVA method (Fig. S1). These results confirmed that aberrant ADt axon formation was due to specific inhibition of the function of Dcc.

DCC Is Required For Correct Asymmetric Growth of the ADt Neurons

To inhibit Dcc function in zebrafish, we injected a morpholino antisense oligonucleotide (dcc-MO) that blocked Dcc protein synthesis [29]. In embryos injected with dcc-MO (6 ng per embryo), the expression of Dcc protein was reduced to levels below the detection limits of our Western blot analyses (Fig. 3A). Photo-conversion of Kaede in dcc-MO injected lhx5:Kaede animals revealed that ADt neurons sent long processes dorsally, in addition to the normal ventrally projecting axons (Fig. 3B). These dorsally projecting processes sometimes occupied lateral positions and extended posteriorly, or they crossed the midline and extended into the contralateral side of the telencephalon (Fig. 3B). Injection of dcc-MO did not affect ADt neuron migration from the medial region to the lateral region of the telencephalon. The dorsally projecting processes were rarely observed in animals injected with a standard control morpholino. To summarize and compare our results, we analyzed each confocal data file and quantified the pixel intensity values of aberrant dorsal processes using custom written ImageJ macro and MATLAB scripts (Fig. S1). To present the quantified results, we assigned phenotypic scores (Grade 0–3) to each embryo based on the pixel intensity value (Fig. S1). Embryos with more pronounced dorsal processes were assigned higher scores (Fig. 3C). Average phenotypic scores (a.p.s) were calculated for each treatment group where a higher a.p.s reflected more severe aberrant dorsal projections. For statistical analyses, we used the non-parametric Mann-Whitney U test to compare the results of two independent observations with identical or equal variance.

Knocking down Dcc Function Causes ADt Neurons to Project Axons Dorsally or to form Multiple Processes

Because our Kaede photo-conversion results could not discriminate whether the ADt neurons extended single aberrant or multiple processes in the dcc-MO injected embryos, we labeled single ADt neurons with a transient transgenic method (Fig. 4A). Plasmids injected into early stage zebrafish embryos are mosaically distributed among the dividing cells, and mosaic expression of transgenes encoded by the injected plasmids may result in random labeling of single or small numbers of cells in the injected embryos [30,31]. We coinjected a plasmid carrying mmy3:Gal4FF and a plasmid carrying UAS:tdTomato, with and without dcc-MO into lhx5:Kaede embryos at the one cell stage. The mmy3:Gal4FF plasmid carries a Gal4 transcriptional activator variant [32] under control of a forebrain specific enhancer element from the emx3 gene [33]. The UAS:tdTomato plasmid carries tdTomato, a red fluorescent protein gene [34] under control of 5 copies of the Gal4 recognition element UAS. By screening the injected embryos for axons that could be traced and assigned to single labeled ADt neurons, we obtained ADt neuronal projection patterns resolvable at single cell resolution (Fig. 4B). Among the labeled ADt neurons in control animals, none had dorsally projecting axons (Fig. 4B, n = 80). In contrast, about 40% (19 out of 50) of the labeled ADt neurons in
Figure 1. ADt neurons project axons ventrally. In this and subsequent figures, the probes used for whole-mount in situ hybridization are listed in the upper right corner of each panel. Developmental stages are indicated in the lower left corners. Lateral, animals mounted in lateral view, rostral to the left; Frontal, animals mounted in frontal view, dorsal to the top. (A) lhx5 is expressed in the anterior dorsal region of the telencephalon. Dashed line marks the telencephalon-diencephalon border. D: dorsal; A: anterior ventral. Scale bar: 100 μm for lateral view; 60 μm for frontal view. (B) BAC modification via recombination methods. Vertical blue bars represent exons of the lhx5 gene. The Kaede expression cassette replaced the first exon of lhx5 gene. pA: polyadenylation signal sequence; Kan: kanamycin resistant marker; FRT, flippase recognition target. (C) Photo-conversion of Kaede in Tg(lhx5BAC:Kaede) transgenic embryos demonstrates ADt neurons project axons ventrally into the AC and SOT. A live Tg(lhx5BAC:Kaede) transgenic animal was mounted in tilted frontal view to reveal the AC and SOT simultaneously. The region of the left telencephalon was selected for photoconversion. D: dorsal; A: anterior ventral; AC: anterior commissure; SOT: supraoptic tract. Scale bar: 60 μm.

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-MO injected animals had either dorsally projecting axons or multiple processes (Fig. 4B). The dorsally projecting axons extended laterally or crossed the midline to extend into the contralateral telencephalon (n = 7). The axons with multiple processes often projected both dorsally and ventrally (n = 12). We further examined all single confocal slices to determine the origins of the axons on the surface of the labeled neurons (Fig. 4B). In control embryos, the origins of the axons were found overwhelmingly on the ventral side of the cell bodies (92%, 74 out of 80). The other 6 labeled neurons either had the axon origins on the dorsal side (4%, n = 3) or close to the middle (4%, n = 3) of the cell bodies. In contrast, for those labeled neurons that had dorsally projecting axons in dcc-MO injected embryos, the origins of the axons were found on the dorsal side of the cell bodies (84%, 16 out of 19). Thus, the single cell labeling results mirrored our results obtained from group labeling, and together these results suggest that Dcc function is required for the asymmetric outgrowth of the ADt axons. Furthermore, because the labeled ADt neurons always extend single axons in control animals, the formation of ADt neuron axons with multiple processes in dcc morpholino injected embryos (Figure 3C) suggest that Dcc function is required for polarized axon initiation of the forebrain neurons in vivo.

Inhibition of Netrin1 Function Affects the Asymmetric Growth of the ADt Neurons

To provide further evidence for the role of Dcc in the asymmetric growth of ADt axons, we examined the effect of knocking down Netrin1, a Dcc ligand. In lhx5:Kaede embryos co-injected with ntl1a and ntl1b morpholinos (referred to as ntl-MO, 6 ng each per embryo), ADt neurons developed aberrant dorsal projections similar to neurons in dcc-MO injected embryos (Fig. 5A, a.p.s. = 0.857, p = 0.820 versus dcc-MO injected embryos). Injection of either ntl1a-MO or ntl1b-MO alone didn’t cause ADt neurons to project axons dorsally. Co-injection of ntl1a and ntl1b mismatch control morpholinos didn’t cause ADt neurons to...
Figure 4. Inhibition of Dcc function causes ADt axons to project dorsally or to form multiple processes. (A) Labeling of individual ADt neurons by mosaic expression of fluorescent protein tdTomato. Image of a live 36 hpf Tg( lhx5BAC:Kaede) animal that was injected with emx3:Gal4FF and UAS:tdTomato plasmids is shown. The tdTomato labeled neuron projected an axon into the AC. Merge panel shows the position of the tdTomato labeled soma (marked by an arrowhead). Scale bar = 50 μm. (B) Injection of dcc morpholino causes ADt neurons to project axons dorsally or to form multiple processes. Labeled ADt neurons are marked by arrowheads in the merged panels. Left panels show an ADt neuron with a normal ventrally projecting axon in a control animal. Middle panels show an ADt neuron with an aberrant dorsally projecting axon in a dcc-MO injected animal. Right panels show an ADt neuron with both ventrally and dorsally projecting processes. Black arrow in the single slice images indicates the origin of the axon on the surface of the cell body. Red bar indicates the middle of the dorsal and the ventral side of the labeled neuron cell body. Scale bar equals to 20 μm in the projected images or 10 μm in the single slice images. (C) Additional examples of ADt neurons with multiple aberrant axons in dcc-MO injected animals. Scale bar = 15 μm.
develop aberrant projections (data not shown). Knocking down both Dcc and Neogenin1 by co-injecting dcc-MO (6 ng per embryo) and neogenin1-MO (3 ng each per embryos) caused the ADt neurons to develop aberrant projections in a manner similar to knock down of Dcc alone (a.p.s. = 1.09, Fig. 3B and Fig. S2). A lower dose of neogenin1-MO (3 ng each versus 6 ng each per embryo) was used in the Dcc-Neogenin1 double knockdown experiment due to concerns over potential toxicity of high dose morpholino injections.

To test the genetic interaction between the Dcc and Neogenin1 knockdowns further, we examined the synergetic effect of Dcc-Neogenin1 morpholino injections at sub-threshold concentrations that did not cause significant aberrant axon phenotype when injected alone [33,36]. We first generated dose response curves for the Dcc and Neogenin1 single knockdowns [Fig. S3]. When a sub-threshold dose of dcc-MO (1.5 ng per embryo) was injected, 9.6±3.7% of the injected embryos developed dorsal axons (n = 60). Injection of a sub-threshold dose of neogenin1-MO (1.5 ng each per embryo) caused aberrant dorsal axons in 11.5±5.4% of embryos (n = 60). These penetrance numbers were not significantly different from the percentage of aberrant axons observed in standard control morpholino injected embryos (9.0±6.4%, n = 55; ANOVA, F2,9 = 1.015, p = 0.393). On the other hand, co-injection of the sub-threshold concentrations of dcc-MO and neogenin1-MO together resulted in the formation of significant number of aberrant dorsal axons (42.1±3.7% of embryos, n = 61; ANOVA, F2,6 = 52.31, p = 1.60×10^{-4}; Fig. 5C). These results indicated that Dcc and Neogenin1 genetically interacted to determine the asymmetric outgrowth of the ADt neuronal axons.

Neogenin1 Knockdown Enhances the Aberrant ADt Axons in dcc-MO Injected Embryos

The guidance receptor Neogenin is closely related to DCC [37,38]. Neogenin1 is expressed in the central nervous system and the mesoderm throughout early development in zebrafish embryos [39,40]. Previous studies showed that knock down of Neogenin1 function by morpholino injection caused abnormal development of the somite and the central nervous system in zebrafish embryos [40,41]. We examined whether inhibition of Neogenin1 function also affected the asymmetric growth of ADt neurons. In embryos injected with neogenin1 morpholino (neo-ATGMO, 2 ng per embryos [40]), the aberrant dorsal ADt neuronal projections (a.p.s. = 0.091) were rarely observed and were not significantly different from those in the standard control morpholino injected embryos (p = 1.000). Injection of higher doses of neo-ATGMO (4 ng per embryos) caused reductions of the AC or both the AC and the SOT (Fig. 5A), whilst it didn’t result in significant aberrant dorsal projections (a.p.s. = 0.053, p = 1.000 versus standard control morpholino injected embryos). Similar to previous report [40], increasing the dosage of the neo-ATGMO further (8 ng per embryo) caused malformation of the neural tube and the ADt neurons (data not shown). When we co-injected both the neo-ATGMO (2 ng per embryo) and the dcc-MO (6 ng per embryo) into the lbo:Kaede embryos (Fig. 5A), the aberrant ADt neuronal projections were enhanced (Fig. 5B, a.p.s. = 1.800, Fig. 5B and Fig. S2). Co-injection of the sub-threshold dcc-MO (1.5 ng per embryo) and the neo-MO (2 ng per embryo) caused a significant increase in the number of aberrant dorsal axons (57.5±9.5% of embryos, n = 58) compared with the single injections (8.1±3.0%, n = 62, in neo-MO injected embryos; ANOVA, F2,6 = 53.49, p = 1.16×10^{-4}, Fig. 5C). These results indicated that Neogenin1 may partially compensate for the loss of the Dcc function in the dcc-MO injected embryos.

Discussion

DCC Regulates Polarized Initiation and Asymmetric Outgrowth of Forebrain Neuronal Axons

Our studies demonstrate that Dcc signaling plays a central role in asymmetric outgrowth of ADt neuronal axons in the zebrafish forebrain. Dcc and its ligand, Neogenin1, are expressed prior to the time that ADt neurons extend their axons ventrally (Fig. 2). Knock down of Dcc function caused ADt axons to extend aberrantly (Fig. 3). These incorrect axonal projections are not likely due to abnormal specification of the ADt neurons, because the neurons migrated to their correct locations and differentiated along their normal time course. Single cell labeling showed that ADt neurons projected axons dorsally or formed multiple axons (Fig. 4). Because the labeled ADt neurons always extend single axons in control animals, the formation of ADt neuron axons with multiple processes in dcc morpholino injected embryos suggests that Dcc signaling is required for polarized axon initiation of the forebrain neurons in vivo. Previous studies in C. elegans showed that the correct asymmetry of HSN neurons was lost in unc-40 (dce) mutants [11] and HSN axons deviated from their correct ventral trajectory to extend dorsally [15]. Thus, our results in zebrafish mirror those observed in C. elegans, suggesting that the functions of Dcc in asymmetric outgrowth of neurons are likely conserved in metazoans.

Previous studies have shown that DCC signaling may promote axonal branching in cultured cortical pyramidal neurons [42], and that disrupting DCC function leads to a decrease in the number of axon terminals in cultured dopaminergic neurons [43]. In Dcc knockout mice, ventral mitral cell axons deviated from their normal path and extended towards more dorsal locations [44]. A recent study showed that cultured hippocampal neurons developed multiple axons when DCC expression was reduced in the context of Bcl11A knockdown [45]. These observations, together with our results, suggest that DCC’s function in neuronal polarity regulation may depend on the particular environment experienced by developing neurons.

Our studies show that DCC, Neitin1, and Neogenin1 play overlapping and differential roles in the asymmetric outgrowth of ADt neuronal axons. Inhibition of the function of either Dcc or Neogenin1 caused ADt neuron to project axons dorsally, whereas inhibition of Neogenin1 function didn’t result in significant dorsal ADt axons (Fig. 5). These results suggest that Neogenin1-Dcc signaling plays a primary role in directing the asymmetric outgrowth of forebrain neuronal axons. Interestingly, knock down of Dcc and Neogenin1 together enhanced the formation of aberrant dorsal ADt axon. This result suggests that Neogenin1 may act in parallel with Dcc and that Neogenin1 may partially compensate for loss of Dcc function in dcc-MO injected embryos. Because knock down of Dcc and Neogenin1 together caused more aberrant dorsal ADt axons than knock down of Dcc and Neogenin1 together, it is likely that other cues in addition to Neogenin1 mediate asymmetric outgrowth of the ADt axons. Neitin2 and Neitin4, two Neitin family member proteins that may interact with Dcc in zebrafish, are not expressed in the forebrain prior to 36 hpf [46] and thus are unlikely to act with Neogenin1 as ADt guidance factors. On the other hand, RGMa, Draxin and MADD-4 (ADAMTL1 and ADAMTL3) are known to interact genetically or physically with Dcc or Neogenin1 [41,47–49]. Further studies are needed to determine whether these additional factors are involved in the asymmetric outgrowth of the ADt neurons.
Figure 5. Effects of inhibition of Netrin1 or Neogenin1 function on the ADt axons. (A) ADt neurons project axons dorsally when Netrin1 function is inhibited (ntn-MO). Knockdown of Neogenin1 function doesn’t cause ADt neuron to project axon dorsally (neo-MO). Images were processed as in Fig. 3B. The pixel intensity value of aberrant axon is shown in the bottom left corner of each panel. Scale bar = 50 μm. (B) Quantitation of ADt neuronal axon defects. Horizontal axis shows the treatment group labels and vertical axis shows the percentage of embryos in each phenotypic category (Grade 0–3) for each treatment group. Numbers inside parentheses denote numbers of animals analyzed for each treatment group. (C) Synergistic effects between sub-threshold Dcc-Netrin1 and Dcc-Neogenin morpholino knockdowns. dcc-sub_MO and ntn-sub_MO: sub-threshold concentration morpholino. At least three independent injections were performed for each treatment group. Numbers inside parentheses denote numbers of animals analyzed. Asterisks represent *p* < 0.001 by ANOVA test. doi:10.1371/journal.pone.0036516.g005
Transgenic Labeling of Forebrain Axons

Few previous studies have examined the roles of DCC in neuronal polarity regulation in vertebrates. We took advantage of transgenic labeling methods to investigate function of Dcc in ADt axon development in zebrafish. The Kaede photo-conversion method allowed specific labeling of ADt axons. Such specific labeling should facilitate future studies of gain and loss of function of other genes that may be involved in ADt axon development. Due to diffraction and scattering of the laser beam in live intact animals, other photo-convertible fluorescent proteins such as KikGR [50], in combination with two-photon conversion may be required to study cell population located in deeper regions.

Our study also utilized single cell labeling by generating mosaic transgenic animals. Single cell labeling provides increased specificity and should be useful in studies of cell migration and neural circuit formation. The mosaic labeling method requires injection and screening of many animals; in our studies, fewer than 10% of the injected embryos yielded useful information and screening by confocal microscopy was time consuming. Although such analyses are much more efficient in C. elegans, where stereotyped neurons can be observed routinely at single cell resolution, further analyses of single cells in vertebrate nervous systems are warranted due to the large numbers of different cell types and neuronal connections.

Materials and Methods

Fish Maintenance

Zebrafish were maintained in a recirculating water system according to standard protocol [51]. Recirculated water was dosed to 500μF salinity with artificial ocean salt mix and buffered to pH7.2 with NaHCO3. Embryos were obtained by breeding adult fish and raised at 28.5°C as described [51]. Embryos were staged by hours post fertilization (hpf) as described [52]. The Fudan University Institutional Animal Care and Use Committee approved all work with zebrafish animals.

Cloning

Zebrafish dce cDNAs were amplified by RT-PCR with primers dce-F1 (5’-CTCCACCATGG GCTGGCTACCGAGG), dce-R1 (5’-GATGAGCGGCACAATCAGGACCAC), dce-F2 (5’-GGGGTCTCGGATAAAGACACACTCATCACC), dce-R2 (5’-GTAACCGCTGATCTGGTGTGATGGGCGTTG). The two overlapping dce cDNA fragments were then assembled into a full length dce cDNA clone. To construct a dce cDNA that was resistant to anti-sense inhibition effects (dce-R-mRNA), silent mutations were introduced into the dce cDNA sequence with forward primer dce-MO-resistant (5’-CTCAAGCTTGAAATTCTCCACATGGGGGTGTG TGCACAGTGACACACGCCC-GAATTTGCGGCTCAC-T, the mutated nucleotides are underlined).

Synthetic mRNA and Morpholinos

Capped mRNA was synthesized with mMESSAGE mMACHINE kit (Ambion). The dosage for mRNA injections was 350 pg of dce-R-mRNA per embryo.

Morpholinos were synthesized by Gene Tools: standard control: 5’-CCTCTTACCTCTCGTT ACAAATTTATA; dce-MO: 5’-GAATATCTCCATGAGCGCAAGGCGCAT [29]; dce-misMO: 5’-CAAATGTTACAGTTACGACCAGCGCAT (mismatch nucleotides underlined); ntl-MO: 5’-CCAAAGCATCAGAGACCTCTCAACAT; ntlb-MO: 5’-CGCAGCTTACAAAATCTGGATCAG [29]; ntl-MO and ntlb-MO were also injected at various doses as indicated in the result section.

Generation of Tg(lhx5BAC:Kaede) Transgenic Line

A zebrafish bacteria artificial chromosome clone (BAC) containing the hlx5 coding regions (cloned id: CH211-230H6) was identified using a genomic database search (http://www.ensembl.org/Danio_rerio/Info/Index) and fingerprinting analysis (http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish/). Targeted modification of the BAC with the photoconvertible fluorescent protein Kaede [22] was based on homologous recombination [23], and microinjection of BAC DNA was previously described [24]. To generate the targeting fragments for homologous recombination, plasmid DNA containing a kanamycin resistant cassette flanked by FRT-sites were used as templates with oligonucleotide primers kare-F (5’-CCCATTCGG-GAGGCGCGGTGCCCGTTTGGGAAGGTCG GCTCTCGGTCCCC TCCAGCGGCGACACATGATCCTGATTAAACCCAG) and kare-R (5’-GGAGATTAGTCCAGAAGTTTGACGAGGTGATG-TG T-GATGGAG). For these primers, nucleotides in italics are homologous to the targeted hlx5 gene, and those underlined are homologous to the plasmid DNA templates. The kanamycin resistant marker was removed by induction of Flpe expression [23]. Homologous insertion of the transgenes and the removal of the kanamycin marker were verified by PCR analysis. Integrity of the modified BAC was checked by fingerprinting analysis. The Tg(lhx5BAC:Kaede) line was maintained by outcross and inbreed generations in our fish facility.

Whole-mount in situ Hybridization

Whole-mount in situ hybridization was performed as described [21]. Digoxigenin-labeled antisense RNA probes were hybridized and then detected with alkaline phosphatase-conjugated digoxigenin antibody Fab fragment (1:7500, Roche) and alkaline phosphatase substrate NBT/BCIP (1:80, Roche). Fluorescent in situ hybridization was performed as described [53]. Digoxigenin-labeled dce and fluorescein-labeled hlx5 antisense RNA probes were detected by Tyramide Signal Amplification kits (Invitrogen). The clones used in this study are: hlx5 [21], dce [29], ntl-MO (accession number: BC114259), and ntlb-MO (accession number: CK60495).All clones were verified by sequencing.

Photo-conversion of Kaede

Photo-conversion of Kaede was performed with a confocal microscope (Olympus FV1000) equipped with SIM module and 405 nm laser. Live embryos were mounted with 1.2% low melting temperature Agarose and the ADt region on one hemisphere of the forebrain was marked with the polygon selection tool of the FruView software (Olympus). Ten pulses of focused 405 nm laser light were then applied to the selected region. Each pulse lasted 500 msec and utilized 60% of the light output of the 405 nm laser. Confocal image stacks were acquired 30 min later after the photoconverted Kaede diffused into the neuronal processes.

Western Blot

Embryos were deyolked in cold Ringer’s solution supplemented with 1 mM EDTA, 0.3 mM PMSF and protease inhibitor cocktail...
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(Stroh), Deyulked embryos were lysed in SDS-sample buffer and heated at 95°C to extract and denature protein. Protein samples were resolved on SDS-PAGE gel then transferred to nitrocellulose membrane (Whatman). Rabbit zebrafish Dcc antibodies (Anaspec) were used at 1:50. The specificity of the Dcc antibodies were verified by cross checking with antibodies against Myc (AbMart) and GFP (Invitrogen) for heterologous expression of Myc- and GFP-tagged Dcc. Endogenous Dcc protein was detected as a band of approximately 170 kDa.

Genetic Single Cell Labeling
A mixture of two plasmids (5.2μg 3Gal4FF and 5xUASsflTo-moto (10 ng/μl each) was injected into one-cell stage embryos. Injected embryos were screened by fluorescent dissection microscopy at 24 hpf and then by confocal microscopy at 36 hpf to identify animals with axons that can be traced and assigned to labeled single cells. To label cells in dcc morpholino injected animals, dcc morpholino was injected at one- to four-cell stage after the plasmid injection.

Microscopy and Image Analysis
Whole mount in situ images were acquired on a dissection microscope with a CCD camera (Leica M205FA). For confocal microscopy, live or fixed embryos were mounted with 1.2% low melting temperature Agarose and imaged on an Olympus FV1000 confocal laser scanning system with 40x water immersion objective. The captured image stacks were converted by ImageJ (http://rsbweb.nih.gov/ij/) for reconstruction with FluoRender software (http://www.sci.utah.edu/software/46-documentation/137-fluorender.html). Reconstructed three dimensional images were projected to a standardized view for figure presentation. To quantify the aberrant projection phenotypes, confocal data files were automatically processed by custom written ImageJ macro and MATLAB scripts (Fig. S1, Text S1, and Text S2). For statistical analysis, non-parametric Mann-Whitney U tests and ANOVA tests were performed with SPSS software. Two-tailed exact p values were reported for the Mann-Whitney U tests and significance P values were reported for the ANOVA tests (SPSS Inc.).

Supporting Information

Figure S1 Quantification of aberrant dorsal axons. (A) Procedure of quantification. The ImageJ macro extracts image stacks, selects the converted Kaede channel, and saves the projections as gray scaled images. The processed images stacks, selects the converted Kaede channel, uses maximum Procedure of quantification. The ImageJ marco extracts image

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